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A high throughput flow cytometry assay for identification of inhibitors of 3', 5'-cyclic adenosine monophosphate efflux

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Summary

Assays to identify small molecule inhibitors of cell transporters have long been used to develop potential therapies for reversing drug resistance in cancer cells. In flow cytometry, these approaches rely on the use of fluorescent substrates of transporters. Compounds which prevent the loss of cell fluorescence have typically been pursued as inhibitors of specific transporters, but further drug development has been largely unsuccessful. One possible reason for this low success rate could be a substantial overlap in pump substrate specificities and functions between transporters of different families. Additionally, the fluorescent substrates are often synthetic dyes that exhibit promiscuity among transporters as well. Here, we describe an assay in which a fluorescent analog of a natural metabolite, 3',5'-cyclic adenosine monophosphate (F-cAMP), is actively effluxed by malignant leukemia cells. The F-cAMP is loaded into the cell cytoplasm using a procedure based on the osmotic lysis of pinocytotic vesicles (1). The flow cytometric analysis of the fluorescence retained in F-cAMP loaded cells incubated with various compounds can subsequently identify inhibitors of cyclic AMP efflux (ICE).

Keywords

cyclic AMP; fluorescent substrates; high-throughput flow cytometry; efflux inhibitors; ABC transporters

1. Introduction

ATP-binding cassette (ABC) transporters are often associated with multidrug resistance in relapsed cancers. ABC transporters are also implicated in the initiation and progression of metastatic malignancies due to their increased expression in cancer stem cells (2, 3). As a result, the development of novel approaches to study ABC transporter activity to identify small molecule modulators or inhibitors is vital for translation to cancer therapy. Previously, high throughput assays for transporter inhibition have relied upon the use of inside-out vesicles containing specific transporters, or of cancer cell lines which have been induced or engineered to acquire drug resistance (4–7). In both cases, these approaches have been

largely dependent on measuring the intracellular fluorescence of synthetic substrates remaining in cells after exposure to compounds. While several small molecules identified in this manner were capable of reducing the multidrug resistance of cancer cells *in vivo*, few of these molecules have been successful in clinical trials (8–10). This may be due in part to the limitations of these transporter efflux assays.

Often, synthetic fluorescent dyes known to be substrates of a particular transporter are used as probes to screen for hit compounds. A major limitation of this technique is the considerable similarity in cell transporter structures, and therefore substrates are capable of being effluxed by a multitude of transporters (11). Thus, the inhibitory compounds identified in these assays fail to exhibit specificity toward the originally investigated transporter. Other attempts at measuring efflux of cellular metabolites have used radioactive conjugates of substrates to evaluate changes in intracellular concentrations. However, this results in lower throughput data collection (12). The approach described here differs from earlier methods because it uses a fluorescent conjugate of a natural metabolite of cell membrane transporters, 3',5'-cyclic adenosine monophosphate (cAMP), and measures its efflux from cells through unmodulated, endogenously expressed transport proteins, allowing for thousands of samples to be measured in a short period of time.

The cAMP signaling pathway has been a focus of cancer research due to its relationship to multiple intracellular signaling components and, specifically, programmed cell death, apoptosis. Existing evidence indicates aberrant cAMP regulation in malignant cells in comparison to normal, healthy cells (13). It has long been known that the increased concentration of intracellular cAMP is capable of triggering cell death in certain cancer cells (14). Typically, such research has relied upon modulation of the major cAMP-synthesizing enzymes adenylate cyclases (AC) or phosphodiesterases (PDEs) that hydrolyze cAMP to increase cytosolic cAMP and reduce cancer cell survival (13, 15). Until recently, one additional step of the cAMP signaling pathway that could be targeted for drug discovery has been overlooked: cAMP efflux by ABC transporters. The approach described here utilizes high throughput flow cytometry for measurement of the efflux of a fluorescent cAMP analog (F-cAMP, Fig. 1) from leukemic cells. This assay assesses the intrinsic ability of cells to efflux cAMP through their endogenously expressed transporters rather than through analysis of the activity of specific protein targets. It thus allows the measurement of the inherent efflux potential of a cancer cell,

As a proof of this concept, we demonstrate the accumulation and release of F-cAMP with flow cytometry (Fig. 2). We present here the results of a high throughput screen (HTS) for the identification of ICE from two libraries: the SPECTRUM Collection (2320 compounds – 60% drugs, 25% natural products, 15% bioactive components) and the Prestwick Chemical Library (~1200 previously FDA-approved drugs). The acute myelogenous leukemia (AML) cell line U937 was screened according to the methods described in this chapter, and the cells were incubated with compounds overnight (~18 h; Figs. 3 and 4). From this screen, 51 hits were identified as having F-cAMP fluorescence 2 standard deviations above the plate mean negative control values. (Fig. 4). These hits were validated by testing the compound F-cAMP efflux inhibition in 8-point dose responses ranging from 60 μ M to 9 nM (Fig. 5A). For this validation, the F-cAMP mean or median fluorescence intensity (MFI) values were

normalized based on F-cAMP fluorescence at time = 0. The dose-response curves were fit with sigmoidal curves, and those compounds which exhibited EC₅₀ values < 30 μ M and had well-behaved sigmoidal dose response curves were selected for further validation (Fig. 5B). This yielded 8 compounds of interest (artemisinin, parthenolide, patulin, quinalizarin, harmalol, pyriithione zinc, clioquinol, and cryptotanshinone). Bioinformatics analysis of the compound structures allowed for the identification of 3 additional related small molecules: artesunate, artemether, and dihydroartemisinin. The 11 compounds of interest were tested in dose response in flow cytometric apoptosis and cell cycle secondary assays to measure the effects of ICE on U937 cell vitality after overnight incubation (Fig. 6). Additional CellTiter-Glo® viability measurements found that 6 of the hit compounds (artesunate, dihydroartemisinin, clioquinol, cryptotanshinone, parthenolide, and patulin) were selectively inhibitory to leukemia cells at EC₅₀ concentrations much lower than those determined for healthy human primary blood mononuclear cells (PBMCs; data not shown). Subsequent testing of the hit compounds to determine mechanisms of action confirmed that the molecules work by modulation of the cAMP pathway and induce programmed cell death. Thus, the utility of the F-cAMP efflux assay approach to identify ICE for leukemia therapeutics has been validated.

2. Materials

All solutions should be prepared following proper aseptic techniques. Dispose of waste materials according to appropriate regulations. All fluid component handling and storage is done in polypropylene tubes: 1.7 mL microcentrifuge, 15 mL conical, 50 mL conical. Cells are grown and incubated in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

2.1. Components for Fluorescent Cyclic AMP Loading

1. 50 mL NF-RPMI: RPMI-1640, 5 mg/L phenol red, 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES. No fetal bovine serum (FBS).
2. 50 mL cRPMI: RPMI-1640, phenol red, 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, and 10% heat-inactivated fetal bovine serum (FBS; *see Note 1*).
3. Fluorescent cAMP analog (F-cAMP), Alexa Fluor®488 8-(6-aminohexyl) aminoadenosine 3',5'-cyclicmonophosphate, bis(triethylammonium) salt (*see Fig. 1 and Note 2*).
4. Hypertonic solution: 10% w/v polyethylene glycol (PEG), 500 mM sucrose in NF-RPMI. 0.1 g poly(ethylene glycol) (PEG) 1,000, 0.17 g sucrose, 0.9 mL NF-RPMI. In a 15 mL conical tube, add 0.9 mL NF-RPMI, then weigh and add 0.1 g PEG and mix thoroughly by vortexing (*see Note 3*).
5. In a sterile tissue culture hood, push the solution through a 3–10 mL syringe fitted with a 0.2 μ m nylon membrane filter (Pall Corporation) and transfer into a sterile 1.7 mL microcentrifuge tube.

6. Hypotonic solution: 40% purified, deionized water, 60% cRPMI. Add 1 mL sterile water to 1.5 mL cRPMI in microcentrifuge tube. Mix by light vortexing. Filter-sterilize as in **step 2.1.5**.
7. Cells known to efflux cAMP, $1\text{--}2\times 10^7$ in a 50 mL conical polypropylene tube (*see Note 4*).
8. 1.7 mL microcentrifuge tube.
9. Centrifuge(s) with capacity for 50 mL conical and 1.7 mL microcentrifuge tubes.
10. T-75 tissue culture flask for F-cAMP loaded cell equilibration.

2.2. cAMP Efflux Assay Components

1. 1 mL untreated cells (no F-cAMP loaded) at 4×10^5 /mL for autofluorescence testing.
2. F-cAMP loaded cells from **step 2.1**.
3. Sterile 12-well cell culture plate with lid (or other means of covering for incubation).
4. 20 mL cRPMI.
5. 60 μ L 30 mM MK571 positive control, solubilized in dimethyl sulfoxide (DMSO).
6. 80 μ L DMSO
7. Flow cytometer with 488 nm laser, 530/30 bandpass optical filter, and multiwell plate auto-sampling capabilities.
8. 15 sample tubes for flow cytometer.

2.3. High Throughput Screening Components

1. 1 mL untreated cells (no F-cAMP loaded) at 4×10^5 /mL for autofluorescence testing.
2. F-cAMP loaded cells from **step 2.1**.
3. Multiwell plates, preferably 384-well, for compound mother plates and the high throughput F-cAMP efflux assay (*see Note 5*).
4. Compound mother plates: 384 well plates which contain positive and negative controls, as well as compounds of interest to be tested in the high throughput F-cAMP efflux assay.
5. 110 μ L 10 mM MK571 positive control per compound mother plate.
6. 225 μ L DMSO negative control per compound mother plate.
7. Proper seals (e.g., DMSO resistant adhesive foil microplate seals) and storage equipment, such as dessicators and/or -20°C freezer, for the compound mother plates (*see Note 6*).

8. Liquid dispensing equipment: this can either be an automated system or multichannel pipettors capable of delivering 5 μ L volumes to wells in 384-well plates (*see Note 7*).
9. 100 nL pintool or analogous compound transfer equipment (*see Note 8*).
10. 384-well plate lids or seals for the assay plates (*see Note 9*).
11. Multiwell plate vortexer.
12. Flow cytometer with 488 nm laser, 530/30 bandpass optical filter, and multiwell plate auto-sampling capabilities.
13. Software which can resolve data from individual wells after high throughput flow cytometry analysis.

3. Methods

3.1. Loading F-cAMP into cells

1. Centrifuge cells at $160 \times g_{\max}$ for 10 min and remove supernatant.
2. Wash/remove serum from cell medium: Resuspend cells in 1 mL NF-RPMI with slow pipetting, add an additional 19 mL NF-RPMI, gently mix, and centrifuge for 10 min. Discard supernatant.
3. While cells are centrifuging, prepare the F-cAMP loading solution. In a microcentrifuge tube, add 200 μ L hypertonic solution and 10 μ L of 5 mM F-cAMP (250 μ M final).
4. Again resuspend the cells in 1 mL NF-RPMI with slow pipetting and transfer to 1.7 mL microcentrifuge tube.
5. Centrifuge the cells at $\sim 120 \times g_{\max}$ for 2 min and remove the supernatant.
6. Gently resuspend the cells in the 210 μ L of F-cAMP loading solution (*see step 3.1.3* above).
7. Incubate the cells with F-cAMP loading solution at room temperature for 10 min, with gentle mixing by hand or 1–2 sec at $\sim 1,600$ rpm every 3 min during this incubation (*see Note 10*).
8. Centrifuge the cells $\sim 120 \times g_{\max}$ for 2 min, remove the supernatant, and resuspend the cells in 1 mL of hypotonic solution.
9. Incubate the cells in hypotonic solution for 2 min at room temperature, with gentle mixing at 1 min.
10. Centrifuge the cells $\sim 120 \times g_{\max}$ for 2 min, remove the supernatant, and gently resuspend the cells in 1 mL of cRPMI.
11. Transfer the 1 mL of resuspended cells to a T-75 tissue culture flask containing 24 mL cRPMI and gently mix. If 10^7 cells were used for the F-cAMP loading, then this gives a final concentration of 4×10^5 cells/mL.

12. Place the T-75 flask in the incubator to allow the F-cAMP loaded cells to recover for 30–120 min (*see Note 11*).

3.2. Optimization of F-cAMP efflux positive control and determining cell F-cAMP efflux ability

This protocol will allow for optimization of the F-cAMP efflux assay and conditions. This step should be performed before the high throughput F-cAMP efflux assay is attempted. Once the positive control and incubation time(s) are determined for a particular cell line, this step may be skipped.

1. Add 500 μ L cRPMI to each well of a 12-well tissue culture plate, then add 500 μ L of F-cAMP loaded cells to all wells and mix by gentle pipetting (final concentration = 2×10^5 cells/mL. Save two 1 mL samples of F-cAMP loaded cells for control purposes, to be used in **steps 3.2.5** through **3.2.8**.
2. Add DMSO and 30 mM MK571 to the wells (Table 1) for $n = 2$ for each concentration tested and mix by gentle pipetting (*see Notes 12–13*). Do not exceed a final concentration of 1% DMSO per sample, as this can be toxic to cells.
3. Incubate the lidded tissue culture plate overnight (16–24 h) under normal cell culture conditions (*see Note 14*).
4. Run control samples immediately after the plate is placed in the incubator, to collect fluorescence data to be used for normalization of the treated samples.
5. Run untreated, non-F-cAMP loaded cells on the flow cytometer, using the 488 nm laser for excitation and collecting fluorescence data with a 530/30 bandpass optical filter. Collect 3,000 events. Create an FSC vs SSC density plot and set a “live cell” gate on the major population of the cells. Then, create an FL-1 channel histogram based on the live cell gate. Data from this untreated sample will provide the baseline/ autofluorescence of the cell line.
6. Run a 1 mL sample of the F-cAMP loaded cells through the flow cytometer with the same settings and parameters detailed in **step 3.2.5** above. Ensure that the FL-1 channel histogram does not go off scale; adjust settings so that the entirety of the histogram is visible (*see Note 15*). This sample will serve as the “100% F-cAMP fluorescence” control for time = 0 of the assay.
7. Place the remaining 1 mL sample of F-cAMP loaded cells at 4°C for the duration of the MK571 optimization plate incubation time.
8. After incubation, run the 4°C-incubated F-cAMP loaded cell sample in the flow cytometer under the same settings used in **step 3.2.5**. This will provide a measurement for the F-cAMP “passive efflux” ability of the cell line (*see Note 16*).
9. Gently mix the contents of each well in the MK571 optimization plate by pipetting, collect individual samples, and interrogate with the flow cytometer (*see Note 17*). Set a gated event count limit for each sample of 1,000 events.

10. To process the F-cAMP efflux data, use MFI values from data collected in **steps 3.2.5** through **3.2.8**.
11. Subtract the autofluorescence MFI value (**step 3.2.5**) from the MFIs of all of the F-cAMP loaded samples, including the F-cAMP fluorescence control collected at time = 0 (**step 3.2.6**).
12. Divide the MFIs from each sample by the MFI collected from the 100% F-cAMP fluorescence/ time = 0 control (**step 3.2.6**). This will indicate the percentage of F-cAMP remaining within the cells under each condition.
13. Fit the MK571 dose response data with a variable slope sigmoidal dose response equation constrained with “bottom” equal to 0 and “top” equal to 100 (fluorescence at initial staining). This will determine the EC₅₀ of MK571 for cAMP efflux inhibition. Example data from an overnight MK571 optimization assay with U937 cells is shown in Fig. 2A.
14. Once an optimal MK571 concentration is determined for a particular cell type, the assay described here may be used to determine the efflux ability of F-cAMP loaded samples at other iterations or incubation times. Example data from an assay to determine the MV411 leukemia cell line cAMP efflux ability is shown in **Fig. 2B**. The MV411 F-cAMP efflux assay data normalized according to **steps 3.2.11** and **3.2.12** is shown in Fig. 2C.

3.3. High throughput F-cAMP efflux assay

The following protocol is designed for a 384-well high throughput assay (*see Note 18*). For information on modifications which may be done to conduct the assay in 96-well plates, *see Note 19*. If no adjustments were made to the protocol completed in **Method 3.1** (25 mL F-cAMP loaded cells at 4×10^5 cells/mL), then this protocol will allow for the creation of 10, 384-well assay plates.

1. Create a compound mother plate with solubilized compounds at 100x final assay concentration. Dedicate wells for negative control (compound solvent) and positive control (MK571; *see Notes 20–21* and Fig. 3 for example plate map). Alternatively, an acoustic dispenser may be used to deliver compounds directly to assay plates in **step 3.3.3**, and if so, the creation of separate compound mother plates is not necessary.
2. Dispense 5 μ L cRPMI to all wells of the 384-well assay plates with a liquid handler or multichannel pipettor.
3. With a 384-well pintool (or other similar liquid transfer equipment), transfer 100 nL from the compound mother plates to the assay plates (*see Note 8*).
4. Dispense 5 μ L F-cAMP loaded cells to the negative and positive control, and compound-treated wells in the assay plates (*see Note 21*). If the added cells were originally at a density of 4×10^5 /mL, then this provides a final cell density of 2×10^5 /mL, or 2000 cells/ 10 μ L final volume well. Save two, 1 mL aliquots of

excess F-cAMP loaded cells for flow cytometer optimization and fluorescence testing.

5. Seal the assay plates and store upside down in the incubator for desired incubation time (*see* **Note 9**).
6. Conduct cytometer optimization and fluorescence testing according to **steps 3.2.5 through 3.2.7**.
7. After assay plate incubation, run the 4°C-incubated F-cAMP loaded cell “passive efflux” sample as in **step 3.2.8**.
8. Vortex (2000 rpm) each F-cAMP efflux assay plate 15s before running samples through the high throughput flow cytometer (*see* **Note 22**). Add an FL-1 vs time density plot to the cytometer collection parameters to allow for well data separation during analysis.
9. Use high throughput flow cytometry analysis software to identify cells and FL-1 MFI from individual wells (*see* Fig. 4 for example data).
10. To analyze well/compound data, exclude samples with < 50 events. Normalize data according to **steps 3.2.11 and 3.2.12** (*see* **Note 23**). Calculate the Z' values for each plate for quality control purposes. Assay plates which indicate a Z' factor of 0.3 should be considered acceptable (16). Hit compounds may be determined per the user’s preference. We identified hits as samples with MFI values 2 standard deviations above the plate mean negative control values.
11. To further validate sample data and decrease the number of false-positive hits, compounds were assayed in a high-throughput dose response assay. Plates were set up as in the HTS, with the exception that the plate formats contained 10-well dose responses for each hit compound, at final concentrations ranging from 30 μ M to 4 nM.

4. Notes

1. If the cells used in this assay are typically cultured in another medium, please substitute that medium for all instances of cRPMI in this protocol.
2. Other fluorescently-conjugated cAMP may be utilized, but fluorophores conjugated to cAMP at sites which do not mimic the molecule shown in Fig. 1 have not been tested nor validated for this assay.
3. Dissolving PEG 1000 in NF-RPMI may be somewhat difficult. Continue vortexing and tilting tube back and forth. If the PEG still does not readily enter solution, wait a few hours or overnight for the flakes to dissolve. Otherwise, placing the tube in a 37°C water bath for 5–15 min may help.
4. We have tested F-cAMP loading in leukemia cell lines, at numbers up to 18×10^6 under the conditions described in this method. For adherent or other cell types, it would be best to optimize the assay starting at cells and gradually testing other cell counts and F-cAMP concentrations to suit your needs. If the assay volumes

and/or cell densities that you require are higher than those described in this protocol, adjust the component volumes and cell numbers loaded with F-cAMP accordingly (see **Note 19**).

5. You will need at least 10, 384-well plates for the cAMP assay, and a number of plates to create compound mother plates. The high-throughput F-cAMP efflux assay protocol described here is for a small assay volume (10 μ L). It would be best to have small volume flat-welled plates or conical wells for these assay plates. In our experience, this assay has worked with both polystyrene and polypropylene multiwell plates.

The compound mother plates need to have the same well format as the assay plates (e.g., 384 wells). The compound mother plates should have well structures optimized for the transfer of small volumes of reagents. We found that wells with conical bottoms were optimal for our 100 nL compound transfers by pintool.

6. The compound mother plates may be saved and reused if stored properly. The plates should be foil-sealed, preferably after being flushed with nitrogen gas to remove excess oxygen and moisture from the wells. The plates can be stored at -20°C (typically best for long-term compound storage, avoiding several freeze-thaws) or in a climate-controlled, low humidity, high nitrogen desiccator. Plates stored in a desiccator may be stable for up to 3 months, depending on the properties of individual compounds.
7. Take note of the “dead volume” specifications of your liquid dispensing equipment. When following the described protocol, ensure that the volumes of the reagents used are adjusted to meet these minimum requirements before beginning the high throughput F-cAMP efflux assay.
8. The wells in this assay will have a final volume of 10 μ L F-cAMP loaded cells in culture medium. A compound delivery of 100 nL will ensure that the DMSO concentration remain below 1%. If a 100 nL pintool or equivalent is unavailable, then intermediate compound dilution plates will need to be prepared to allow for 1 μ L volumes to be transferred to assay wells while maintaining DMSO at 1%.

To prepare intermediate compound dilution plates, stock from the compound mother plates is diluted 1:10 in culture medium. This is accomplished in additional 384-well plates, by combining 9 μ L of culture medium with 1 μ L of the well contents from the compound mother plates. Mix well contents thoroughly by pipetting or plate vortexer before making 1 μ L transfers from the intermediate compound dilution plates into the assay plates (**step 3.3.3**).

9. Depending on the optimal culturing conditions for the cell line tested, the assay plates may be incubated with fitted lids or sealed with gas-permeable, solid polymer, or foil seals. Take into consideration that the assay plates will contain small volumes (10.1 μ L final), and incubation with lids or gas-permeable seals may result in some evaporation of well volumes, especially on the edges of the assay plates. We found that leukemia cell line responses to the F-cAMP efflux assay were best when non-permeable seals were used on the assay plates. Gas

permeable plate covers seemed to decrease cell viability, as evidenced in population shifts on flow cytometer FSC vs SSC density plots when plates were analyzed after incubation.

10. We have not tested the sensitivity of F-cAMP loaded cells incubated and equilibrated in full-light conditions. It is best to incubate the cells in the dark to minimize the potential for ambient light to reduce treated cell MFI's.
11. It is possible for the cells to be used in the efflux assay immediately, but because the plasma membranes have been subjected to stress from the osmotic lysis of pinocytic vesicles in the F-cAMP loading procedure, it would be beneficial to allow the membrane integrity to be regained before proceeding. This step allows the cells to recuperate before testing with compounds. Different cell lines will recover at different rates. Check the cells with a light microscope to determine whether the cells appear healthy and rounded before advancing to the efflux assay.
12. MK571 is a known inhibitor of ABC C-family transporters (also known as multidrug resistance proteins), and it is often used as a positive control for cAMP efflux. The concentrations tested here are used to generate a dose response curve to determine the EC₅₀ of MK571 cAMP efflux inhibition. Alternative concentrations may also be tested. With U937 cells, we determined an MK571 EC₅₀ of ~40 μ M, and used a final concentration of 100 μ M in our HTS assays to ensure effective cAMP efflux inhibition (Fig. 2A).
13. Because this optimization step does not require many F-cAMP loaded cells, multiple plates, MK571 concentrations, and incubation times may be tested as well.
14. The MK571 optimization incubation should be for the anticipated amount of time in which the HTS assay will be completed. We have conducted this assay at varying times from 16–48 h. Please note, however, that cells which do not actively efflux cAMP well, or too short an incubation time may not produce data with any significant effects or dose-dependent efflux inhibition by MK571. Incubation times which are too long (~48 h) could lead to increased levels of apoptosis, depending on the sensitivity to the cells to high concentrations of MK571.
15. If the FL-1 channel cytometer settings had to be adjusted in this step, run the untreated, non-loaded cells (**step 3.2.5**) again to ensure proper assessment of the cell line autofluorescence for the assay readings.
16. For some cell lines, incubation at 4°C can cause apoptosis, and this is evident in shifting of the population on the FSC vs SSC plot. Only use data from events which fall within the “live cell” gate created with the untreated/ autofluorescence cells.
17. All or partial aliquots of the wells may be collected during this step. If only a few hundred microliters of each well are sampled, this allows for the remainder of the

plate to continue to be incubated, allowing for aliquots to be collected at multiple time points.

18. All plate setup and compound handling for HTS can be done in ambient conditions outside of a tissue culture hood.
19. Considerations for completing the high throughput F-cAMP efflux assay in 96-well plates: a) The compound mother plates and assay plates should have the same well configurations, to ease compound transfer. b) Assays conducted in 96-well plates typically require higher minimum volumes per well for samples to be collected by high throughput flow cytometry. These minimum volumes are contingent on the geometry of the plate wells, of course, but it can be expected that the wells would require 50 μ L final volumes. c) While the volume per well can vary from the 10 μ L final volume described in this protocol, it is ideal to maintain final well concentrations of 1% DMSO and 2×10^5 cells/mL.
20. In this step, any number of compound mother plates may be created for use with the 10 assay plates. Therefore, individual compound mother plates may be used multiple times to increase the sample size tested per compound, or each assay plate may be tested with different compound mother plates. For our tests to identify “inhibitors of cAMP efflux”, our compound mother plates consisted of 6 μ L final volume per well. There were 10 mM DMSO-solubilized compounds in all wells in columns 3–22, the negative control was in columns 1–2, and the positive control was in column 23 of the 384-well plate (Fig. 3). Alternatively, if compounds of interest are already known, the compound mother plate can be made to test the compounds in dose response, with negative controls in columns 1 and 13, and dose series in columns 2–10 and 12–22. The MK571 positive control can be incorporated into the plate as a dose response series, or as few wells in column 23 at a static concentration.
21. For example, if the compound mother plates were set up as shown in Fig. 3, F-cAMP loaded cells would be added to all wells in columns 1–23 of the assay plates. Because column 24 would not have any compound or cells added, these wells would serve as “wash” wells.
22. In our assay, we used flow cytometers configured with HyperCyt® autosampling systems (IntelliCyt), with peristaltic pump speeds at 15 rpm, and aspiration for 1 sec/well (up times between wells could be set from 300–500 ms). This allowed for approximately 2 μ L (~400 cells) to be sampled from each well.
23. In some instances, the positive and negative controls data may indicate a gradient of MFI values dependent on well position on the plate. In such cases, it would help to normalize F-cAMP efflux for the samples on a per-row basis (e.g., using only the positive and negative control MFI values from Row H to normalize the Row H sample MFIs).

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Fig. 1.

Structure of the fluorescent cAMP analog (F-cAMP) used in this assay, Alexa Fluor®488 8-(6-aminohexyl) aminoadenosine 3',5'-cyclicmonophosphate, bis(triethylammonium) salt.

Fig. 2.

Cell line MK571 optimization and determination of cell F-cAMP efflux ability. **A)** Example of MK571 optimization assay results. Data shown were obtained using the acute myeloid leukemia (AML) cell line U937 loaded with F-cAMP and incubated overnight (~ 16 h) in appropriate concentrations of positive control MK571. Data was fit with a sigmoidal dose response formula, and constrained with top = 100 and bottom = 0 to determine the EC₅₀ of inhibition by MK571. **B)** Determination of ability of AML cell line MV411 to efflux F-cAMP. Data shown are raw FL-1 MFI values collected from control and treated samples. **C)** Normalized data for the assay data shown in (B). Cell line autofluorescence was subtracted from sample MFI's and normalized such that F-cAMP MFI at time 0 = 100%. This data indicates that MV411 cells lose ~8.5% of F-cAMP through passive leakage (4°C) and ~45% by active efflux (37°C). The positive control with MK571 limited F-cAMP loss to 13%.

Error bars in (B) and (C) indicate the data mean $\pm$ standard error of the mean (SEM) from three independent experiments.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
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Fig. 3.

Sample configuration for a 384-well compound mother plate, as described in **Note 15**.

Solubilized reagents are added to the plate at a minimum volume of 6 μ L. Test compounds may be used in a single-point or dose response manner. This plate incorporates both negative (compound solvent) and positive (MK571) controls so that normalized ability to efflux F-cAMP and Z' values can be determined for each plate. If reagent availability is limited, fewer wells per plate may be used for positive controls.

Fig. 4.

Sample data from a 384-well F-cAMP efflux assay plate collected as in **step 3.3.9**. Data were analyzed with HyperView software (IntelliCyt, Albuquerque, NM, USA) and time-gated to define data from each well. **A)** FSC vs SSC plot with gate around untreated ("live") cells. **B)** FL1-H histogram. **C)** Time vs FL1-H plot from one 384-well plate. **D)** A magnified row from the time vs FL1-H plot in (C), indicating controls and the potential hit compound clioquinol.

Fig. 5. Sample data from hit compound dose-response validation. **A)** Magnified time vs FL1-H plot for an F-cAMP efflux 8-point dose response for the hit compound of interest, clioquinol, including negative and positive controls. **B)** Graph of normalized response for the data shown in (A). Sample FL1-H MFI were normalized such that F-cAMP fluorescence at the time of compound addition = 100%. Data were fit by sigmoidal curves with Hill slope = 1 and top < 250. Graph, fit, and EC₅₀ determination were done with GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA).

Fig. 6.

Results of ICE hit compounds after overnight incubation on U937 leukemia cell vitality in flow cytometric secondary assays. **A)** Dose-dependent effects of the identified ICE on apoptosis. Bars indicate the percentages of cell populations which stained double-positive with Annexin V-PE and 7-AAD, indicating that late apoptotic events had occurred. **B)** Effects of ICE on U937 cell cycle. The bars indicate the percentages of the cell population in each phase of the cell cycle, as determined by propidium iodide (PI) DNA staining. The gating was done on PI histograms as follows: G₁/G₀: DNA = 2n, S: 2n < DNA < 4n, G₂/M: DNA = 4n, Apoptosis (A): DNA < 2n. Error bars in both graphs indicate the data mean $\pm$ SEM from three independent experiments.

Table 1.

Volumes of MK571 and DMSO added to each 1 mL well containing F-cAMP loaded cells to give final DMSO concentration = 1%.

Final conc. MK571 (μM)	30 mM MK571 (μL)	DMSO added (μL)
0	--	10.0
10	1.0	9.0
25	2.5	7.5
50	5.0	5.0
75	7.5	2.5
100	10.0	--