Introduction

A variety of molecular targeted drug screening assays have been developed by the Developmental Therapeutics Program in collaboration with various investigators. A number of additional assay are currently under development. These vary in format, ranging from simple cell-free assays to those involving cell lines engineered to express particular reporter systems. The screening assays are intended for use in conjunction with chemical libraries to support discovery of novel molecular targeted agents, or drug leads, relevant to treatment of cancer, AIDS-associated malignancies, HIV-1 disease or opportunistic infections. For a recent review of the process, see Shoemaker, et al. Application of High-Throughput, Molecular-Targeted Screening to Anticancer Drug Discovery. Current Topics in Medicinal Chemistry 2:229-246, 2002. A downloadable copy of this review is available at: <http://spheroid.ncifcrf.gov/stb/staff/shoemaker/shoemaker.cfm>.

Data generated during assay development (screening data on the NCI Training Set) and results from high-throughput screening of the NCI Diversity Set are available on this page.

C/EBP AS A TARGET FOR DEVELOPMENT OF NOVEL CANCER THERAPEUTICS

- The transcription factor C/EBP plays key roles in regulation of differentation of various cell lineages (adipocytes, keratinocytes, etc.)

- Mutations in CEBP (the gene coding for C/EBP) are associated with development of AML [t(8;21) - subtypes M1 and M2]

- CEBP knock-out mice show no mature neutrophils

- Conditional expression of CEBP is sufficient to trigger neutrophilic differentiation

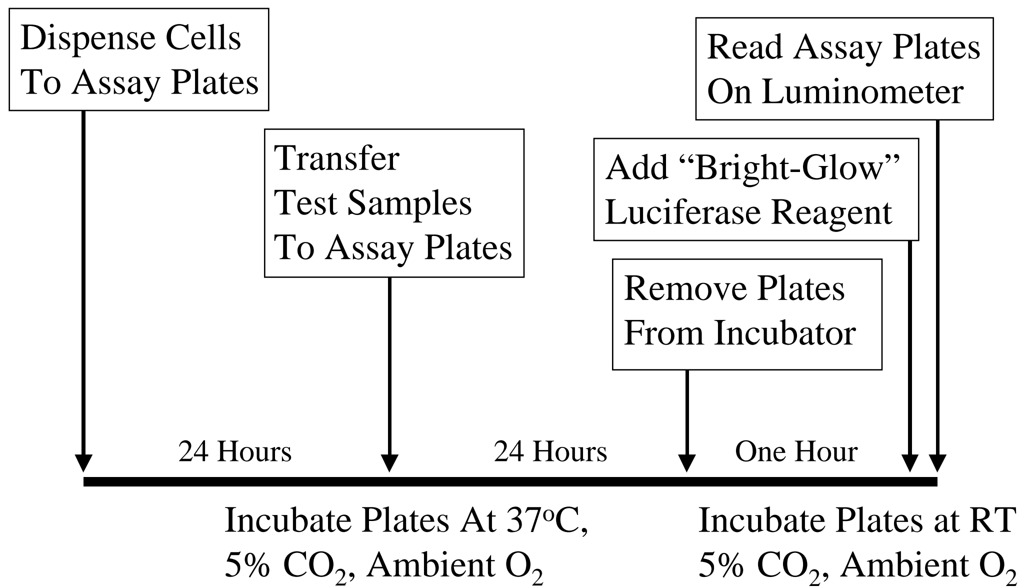
- Pharmacologic modulators of CEBP could act as differentiation inducers and thus limit proliferation of AML cells

References

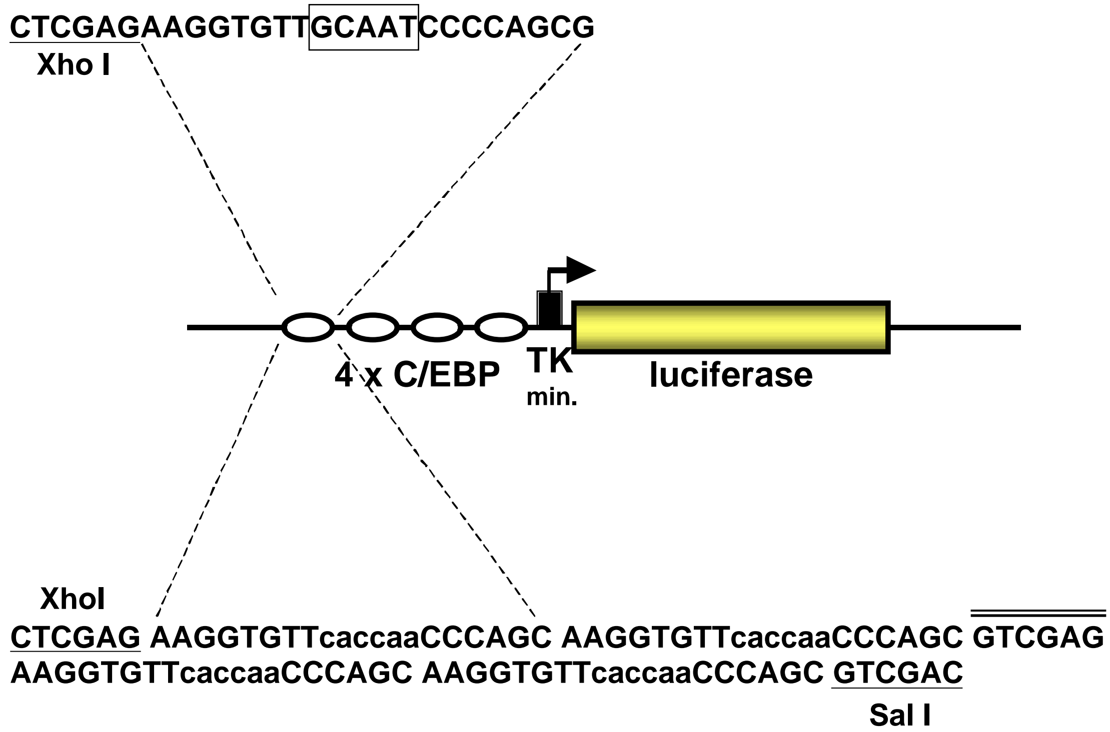
1) c-Myc is a critical target for c/EBPalpha in granulopoiesis
Johansen, L. M. Iwama, A., Lodie, T. A., Sasaki, K., Felsher, D. W., Golub, T. R., Tenen, D. G. **Mol. Cell. Biol** 21 (11) 3789-806 (2001).

2) Abnormalities of the CEBP alpha transcription factor: a major target in acute myeloid leukemia. Tenen, D. G. **Leukemia** 15 (4) 688-9 (2001).

Schematic Protocol for CEBP Alpha Targeted HTS Assay



CEBP Reporter construct



A C/EBP - LUCIFERASE REPORTER ASSAY USING U937 CELL LINE BACKGROUND

STANDARD OPERATING PROCEDURES

I. CELL RECOVERY:

A. U937-C/EBP - regular cell culture protocol is followed. Pull repository vial, thaw, and place in conical tube and pellet. Decant supernatant and re-suspend in T-75 flask containing 25 ml media (RPMI phenol red-free) containing 1% L-Glutamine, 10% FBS and G418 at 1000 μg/ml final concentration for selective propagation.

II. CELL MAINTAINCE:

A. U937-C/EBP - Tissue culture splits are done weekly. 45 ml media are added to T150 flasks. Cells are added to each at a 1:20 or 1:40 ratio.
B. 1 flask is used for each test setup.
C. Maximum passage of 15 generations should be used pending demonstration of loss of gene expression. Backup flasks will be maintained in parallel utilizing a lower (7-10) passage generation.

III. INOCULATION:

A. U937-C/EBP . Pour contents from flasks into 50 ml conical tube. Spin at 1000rpm for 5 minutes to pellet cells. Decant supernatant and re-suspend in 15 ml phenol red-free media with 10% FBS without G418.
Count using hemocytometer. Calculations are made for 20,000 cells/well in 384 well plates.
   a. 25 μl of the cell solution is added to each well of the 384 well plates (white flat bottom COSTAR 384 for the luminescence assay. The number of plates used is pre-determined by the volume of testing.
B. U937 plates are then incubated in 5% CO_2 and 37 ˚ C for 24 hours

IV. FABRICATION OF 384 COMPOUND PLATES: Proposed Plate Replication Scheme for HIF/CEBPa Assays

1. Plates are shipped to the Natural Products Support Group lab. Plates contain 20μl of a 1mM solution.
2. Plates will be replicated on the Apricot 96-tip robotic system (Personal Pipettor 550) as follows:
   a. Parent plate will be placed on deck in position 4.
   b. 384 well Daughter plates will be placed in a plate stacker.

c. Disposable tips will be put on the Apricot.
d. Isopropanol will be placed in solvent reservoir in position 1.
e. Deep well dilution plate will be placed in position 2.
f. Parent plate will have the barcode read.
g. Apricot will add 390µl of Isopropanol to the dilution deep well plate.
h. Apricot will remove 10µlfrom the parent plate and move to the dilution plate and mix the solution well (10µl in 400ul).
i. 384 well Daughter plates will be moved to Apricot deck and the barcode read, the first plate will be used to look up in ORACLE for which quadrant to put the plate into, subsequent plates will be checked and assigned.
j. Either 20 or 40µlof this solution will be transferred to the daughter plate.
k. Plates will be re-stacked to a wait drying.
l. Plate maps will be assigned to ORACLE for importing into plate table.

3. Step 2 will be repeated four times for each set of 384 well daughter plates (four parent plates/set of 384 well daughter plates).
4. Daughter plates are dried under vacuum with a residue of 0.5µl of a 1 mM solution in DMSO remaining.

V. ADDITION OF COMPOUNDS:

A. Thaw 384 well block drug plate at room temperature and place on shaker on low setting to re-suspend.
B. Prepare RETINOIC ACID (NSC 122758) positive controls from frozen stocks. RETINOIC ACID is frozen at a stock concentration of 8mM. Working stock solutions are then diluted to a concentration of 2.5µM in phenol red-free media. Two additional dilutions of the working stock are made (1.25 and 0.625µM). These concentrations, when added to test plates (1:2), yield 3 final plate concentrations of 1.25, 0.625 and 0.313 µM respectively. Each 2x concentration is placed in the appropriate Biomek 6 chambered trough according to stage format for the biomek 2000.
C. Phenol red-free media is added to a separate trough for use in the program: all troughs are placed on the Biomek work surface.
D. Along with the troughs, the thawed block compound plate, 384 well luminescence plates (U937-C/EBP) are taken from incubator and are placed on the work surface.
E. Initiate custom designed program sequence, which will begin by adding media to the control (cells only) column of the 384 well plates (See Plate Format). The block plate containing 0.5µl of sample at a concentration of 1000 µM is diluted to a concentration of 2 µM by adding 249.5µl of media to each sample well.
F. RETINOIC ACID drug controls are added in order to the 384 well plates (see plate format). Using p250 tips, the test compounds are added to

appropriate 384 well test plates in the following manner: 25µlof the 2µMtest concentration are added to each 384 well test plates, as illustrated in plate map format, containing 25µlof U937 cells creating the final 1µM final test concentration.

G. The U937 plates are removed and stored in 5% CO_2, 37 degree incubator for 12-24 hours.

VI. READING:

A. If using a fresh bottle of Bright-Glo substrate buffer thaw buffer at room temperature and mix with lyophilized substrate from kit. (See kit instructions) Store at –20 ˚ C
B. If using a bottle that has been stored in the freezer, thaw the bottle using water at room temperature. Approximately 2 hours.
C. Remove plates from incubator and let equilibrate 1.5 hours at room temperature in 5% CO_2 incubator.
D. After plates and reagent have reached room temperature pour 35 ml of the Bright-Glo substrate into a trough on the work surface, and initiate robotic addition of 40 µl Bright Glo to each well of the 384 well luminescence test plates for a total volume of 90 µl in each well (Bright-Glo must not be warmer than room temperature).
E. Plates are left at room temperature in laminar flow hood for at least 3 minutes and are then read in sequential order using the WALLAC 1420 Reader in Luminescence mode following the custom protocol labeled 100 Milli.
F. Repeat steps D and E for each luminescence plate.
G. Once all Luminescence plates are read, Data is saved and a copy made to a Zip disk. Once copy is verified data is imported into custom EXCEL spreadsheet.
H. Data is then imported into ACCESS database for review.

VII. Analysis

1. The following parameters and descriptions are used to analyze and review C/EBP data.
2. Experimental Data (Avg.& Std.)
   - Cells Only
   - Cells & Optimal concentration of Retinoic Acid (Best of three concentrations)
   - Cells & Test compound
3. Calculated Fields
   - Retinoic Acid Induction = Optimal Retinoic Acid / Cells Only
   - Test Induction = Test / Cells Only
   - Fold Induction = Test Induction / Retinoic Acid Induction
   - Z Prime = 1 – ((3*Std Cells Only + 3*Std Cells & Ret. Acid) / ABS(Avg Cells Only – Avg Cells & Ret. Acid))

4. Quality Control
    - Retest Plate if Retinoic Acid Induction is < 1.5
    - Surface maps are generated to assess potential active compounds and toxicity related to drug profiles. Any surface maps that are deemed inconsistent are flagged for resubmission and confirmation.
5. Growth (for Diversity, Training and Titration Retest)
    - Cells Only
    - Cells and Retinoic Acid
    - Cells & Test Compound
6. Calculated Fields
    - Percent Growth = Test / Cells Only* 100