Standard Operating Procedures: HTS384 NCI60 – NCI-60 One-Concentration Screen

General Description:

As of early 2007, all compounds submitted to the NCI-60 Human Cancer Cell Line Screen are tested initially at a single high concentration (10^{-5} M) in the full NCI-60 cell line panel. Only compounds that satisfy pre-determined threshold inhibition criteria in a minimum number of cell lines will progress to the full Five-Concentration Screen. The threshold inhibition criteria for progression to the Five-Concentration Screen was selected to efficiently capture compounds with anti-proliferative activity based on a careful analysis of historical DTP screening data. The threshold criteria may be updated as additional data becomes available.

Interpretation of One-Dose Data:

The One-Concentration Screen data will be reported as a mean graph of the percent growth of treated cells and will be similar in appearance to mean graphs from the Five-Concentration Screen. The number reported for the One-Concentration Screen is growth relative to the vehicle control, and relative to the number of cells at time zero. This allows detection of both growth inhibition (values between 0 and 100) and lethality (values less than 0). This is the same as for the Five-Concentration Screen, described below. For example, a value of 100 means no growth inhibition. A value of 40 would mean 60% growth inhibition. A value of 0 means no net growth over the course of the experiment. A value of -40 would mean 40% lethality. A value of -100 means all the cells are dead. Information from the One-Concentration Screen mean graph is available for analysis.

NCI 60 Cell Five-Dose Screen

Compounds that exhibit significant growth inhibition in the One-Concentration Screen are evaluated against the 60-cell line panel at five concentrations.
The sixty human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. To initiate a screen, 40 µl of cells are inoculated into the wells of white 384-well microtiter plates at plating densities ranging from 250 to 2,500 cells/well, depending on the doubling time of individual cell lines. After inoculation, the microtiter plates are incubated at 37°C with 5% CO^2 and 95% relative humidity for 24 h before the addition of controls and test agents. Prior to a screen, the test agents and controls are solubilized in dimethyl sulfoxide (DMSO) at 400-fold the final test concentration and stored frozen in polypropylene 384-well microtiter plates.
Twenty-four hours after cell line inoculation, acoustic dispensing is used to transfer 100 nl of DMSO (0.25% (v/v), final) into the wells of microtiter plates, each containing a single cell line. Subsequently, 40 µl of CellTiter-Glo are dispensed into the wells, according to the manufacturer's protocol. Luminescence is measured to assess cell viability at the time of drug addition (time zero, Tz). Acoustic dispensing is also used to transfer 100 nl of controls and test agents (400-fold dilution, final) into duplicate microtiter plates for each cell line to achieve technical replicates. Controls in each microtiter plate include vehicle, 100% cytotoxicity (1 µM Staurosporin NSC755774 and 3 µM Gemcitabine NSC613327 [n = 8]), and five concentrations of doxorubicin

(NSC123127, 25 µM [n = 2], 2.5 µM [n = 1], 250 nM [n = 2], 25 nM [n = 1], 2.5 nM [n = 2]. Following the delivery of controls and test agents, the microtiter plates are incubated for 72 h at 37°C with 5 % CO^2 and 95% relative humidity. After 72 h of exposure, 40 µl of CellTiter-Glo are dispensed into the wells of the microtiter plates and luminescence is measured, according to the manufacturer's protocol, to assess cell viability. Using the various measurements (time zero [Tz], vehicle control growth [C], and growth in the presence of test agent at the five concentrations [Ti]), the percentage growth (%G) is calculated at each of the test agent concentrations. Percentage growth is calculated:

If Ti >/= Tz, the following equation is used:
%G = [(Ti - Tz)/(C - Tz)] x 100
If Ti < Tz, the following equation is used:
[(Ti - Tz) / Tz] x 100

Three response parameters are calculated for each test agent. The GI50 (50% growth inhibition) is calculated from [(Ti - Tz) / (C - Tz)] x 100 = 50, which is the concentration resulting in a 50% reduction in growth compared to the vehicle control-treated cells during the 72-hour exposure period. The concentration resulting in total growth inhibition (TGI) is calculated from Ti = Tz. The LC50 (50% lethal concentration) is calculated from [(Ti - Tz) / (C - Tz)] x 100 = -50. Values are calculated for each of these three parameters if the level of activity is reached; however, if the activity is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.
The NCI-60 screen is performed in two stages: first, a single concentration of 10^{-5} M or 15 µg/ml is tested in all 60 cell lines. If the results obtained meet the selection criteria, then the compound is tested again in all 60 cell lines at 5 x 10-fold dilutions with the top concentration being 10^{-4} M or 150 µg/ml. Compounds accepted for NCI-60 testing are prepared for both single and five-concentration testing at the same time.

Compound Requirements

Two factors influence the quantity of material required and used for each solubilization:

1. Screening Concentration Requirements
2. Screening Volume Requirements

Concentration Requirements

Synthetic agents for the NCI-60 screen with a known molecular weight are prepared in DMSO (unless otherwise noted) at a concentration of 4 mM for the One-Concentration Screen and 40 mM for the Five-Concentration Screen. In both cases the solution is diluted 1:400, giving a high-test concentration of 10 or 100 µM respectively. Synthetic agents (macromolecules or others) without a molecular weight are prepared in DMSO (unless otherwise requested) at a concentration of 6 and 60 mg/ml and diluted 1:400, giving high-test concentrations of 15 and 150 µg/ml.
The goal of solubilization is to deliver the highest requested concentration of an agent for the screening process. For the NCI-60 screening lab, if the amount of material sent is insufficient the test concentration must be decreased to ensure an appropriate volume is met. Crude natural product extracts that are organic solvent

soluble are prepared in DMSO, while those that were produced by aqueous extraction are solubilized in water, both at 40 mg/ml.

Volume requirements

The NCI-60 screen requires a minimum volume of 280 µl, enough for the initial one-concentration screen (75 ul at 4 mM) plus a potential test and retest in the five-concentration screen (each 100 ul at 40 mM). One-Concentration Screening is done at 1/10th the high concentration of the Five-Concentration Screen, so the volume requirement is:

210 µl + 33% at 40 mM = 280 µl for compounds with molecular weights, or
210 µl + 33% at 60,000 µg/ml = 280 µl for compounds without molecular weights (macromolecules) (i.e. less than 10 mg for MW = 1000 or 15 mg for compounds tested as weight/volume).

Compounds Received

Synthetics & Pure Compounds (with known molecular weight or macromolecules and compounds without molecular weights) – Aliquots of agents identified for testing by DTP staff are weighed into glass vials and labeled on the side and bottom with identification barcodes by staff at the Storage Contractor. Prepared vials are transported to Frederick National Laboratory via courier service. Upon receipt, the contents of each package are checked against the accompanying shipping list. Compounds are not re-weighed and are solubilized directly in these vials. Except when specifically noted, all agents are stored in -70 or -20 °C freezers.
Crude Natural Products – Crude Natural Product extracts are selected by the Natural Products Branch staff and plated on detachable polypropylene (PP) 96-well microtiter plates. Plates are dried and stored at the Natural Products Repository at -20 °C until called up for single-concentration testing. Based on the results of the single concentration test, those samples selected for 5-concentration testing are cherry-picked and serially diluted in a 96-well plate.
Compounds with Special Instructions – The special storage or testing instructions provided by the compound supplier can change the handling of the agent and are followed accordingly (e.g. oxygen or light sensitive, no DMSO, prepare fresh, etc.).
Compounds that are identified as needing to be prepared fresh before use are solubilized and serial diluted no more than three hours before use, then immediately transferred to an Echo source plate and stored under nitrogen in a desiccator box until delivered to the testing lab.

Solubilization and Plating Standard Operating Procedures

Entering Information into the NPSG Robotic System

A batch of 88 or 96 compounds is assigned to a unique Plateset by the NPSG Tecan Fluent. Each vial's barcode is scanned, read, and loaded into TECAN software, which looks up the quantity and MW (from NPSG ORACLE tables) and calculates the volume of solvent to be added to each vial to get constant concentration (40mM or 60,000 µg/ml). Adjustments to concentrations are made if insufficient material is available for a standard single concentration screen and two five-concentration screens (initial & retest). A Platemap, an output file that

defines compound to well, is uploaded to NPSG ORACLE tables and DIS PLATES tables for each completed Plateset.

Supplies, Equipment, & Methodology

Vials are loaded on the TECAN Fluent deck in Shiplist order. The robot picks up a vial, reads the barcode, queries the database, and adds the appropriate volume of solvent (DMSO) to each vial to achieve a constant concentration (40 mM or 60,000 µg/ml). The TECAN vortexes and assesses the turbidity of each vial before placing them in 24-position racks. If flagged due to high turbidity, a technician inspects the vial individually and sonicates, warms, etc. to achieve a solution keeping the time of exposure less than two hours. While in the racks, vials then have their bottom barcodes scanned to verify identity and position. Once confirmed the rack is then placed on the TECAN deck in a slanted orientation to facilitate a successful transfer of solutions from vial to plate. A dilution plate is made for single concentration testing at 4 mM or 6,000 µg/ml and the remaining sample is transferred to a 2D barcoded storage tube in a 96-position rack for interim storage for potential five-concentration screening.
For One-Concentration Screening: Dilution plates containing 88 compounds are stored for Echo 384-well source plate preparation.
For Five-Concentration Screening: When compounds have completed the single concentration screening and meet the selection criteria for five-concentration screening, they are grouped in sets of 16, cherry-picked out of the racks of 2D barcoded storage tubes, and serial diluted in a 96-well plate.
Echo source plate preparation: Echo source plates are prepared with three controls in columns 1 and 2: DMSO for high control, a mixture of 1 µM Staurosporin (NSC755774) and 3 µM Gemcitabine (NSC613327) [n = 8] for 100% cytotoxicity, and five concentrations of doxorubicin (NSC123127), 25 µM [n = 2], 2.5 µM [n = 1], 250 nM [n = 2], 25 nM [n = 1], 2.5 nM [n = 2]; and 352 test wells. A combination of 4 dilution plates containing either 88 single concentration agents or 16 five-concentration and eight single concentration agents are transferred to two identical copies of Echo source 384-well plates (35 ul each) using a 96-tip head on the TECAN Fluent.

Vehicle Selection – Synthetics

The vehicles of choice are DMSO and water. Most agents are solubilized using one of these two vehicles. Other vehicles are used at the request of the compound supplier or based on past testing methods. Agents utilizing volatile solvents as a vehicle are labeled 'Fresh' and are prepared within an hour of screening addition. Currently, all synthetic agents for NCI-60 screening are prepared in DMSO, unless another vehicle is indicated. When water is indicated, the compound is solubilized in either distilled water or saline.

Solubility Codes

The agents will not always solubilize to a clear solution absent of particles. Therefore, the solution is identified via a code best describing the solubility of the agent in the vehicle. Special Note: It is the clarity of the solution that is being evaluated. The presence or absence of color is not accounted for in the solubility codes.