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COMPETITION ASSAY

Membrane preparation

Frozen tissue or washed cells are partially thawed and homogenized in 20 volumes of cold lysis buffer (50mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail). After a low speed spin (1,000 x g for 3 min) to remove large tissue chunks, the homogenate is centrifuged at 20,000 x g for 10 minutes at 4°C to pellet the membranes. The pellet is resuspended in fresh buffer and centrifuged at the same speed a second time. The pellet is then resuspended into buffer (15 ml) containing 10 % sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay. On the day of the assay the membrane preparation is thawed and the pellet resuspended in final assay binding buffer (50 mM Tris, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4).

Incubation and filtration

The filtration binding assay is carried out in 96-well plates in a final volume of 250 µL per well. To each well is added 150 µL membranes (3 - 20 µg protein for cells or 50 - 120 µg protein for tissue), 50 µL of the competing test compound and 50 µL of radioligand solution in buffer. The plate is incubated at 30 °C for 60 minutes with gentle agitation. The incubation is stopped by vacuum filtration onto 0.3 % PEI presoaked GF/C filters using a 96-well FilterMate™ harvester followed by four washes with ice-cold wash buffer. Filters are then dried for 30 minutes at 50°C. The filter is sealed in polyethylene, scintillation cocktail (Betaplate Scint; PerkinElmer) added and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

Data analysis

For each drug concentration, non-specific binding is subtracted from total binding to give specific binding. Data is fitted using the non-linear curve fitting routines in Prism® (Graphpad Software Inc). For competition assays, Ki values are calculated from IC_{50} values using the formula $K_i = IC_{50} / (1 + ([S]/K_d))$ where [S] is the radiotracer concentration used in the assay and K_d is the dissociation constant of the radiotracer.

SATURATION ASSAY

Membrane preparation

Frozen tissue or washed cells are partially thawed and homogenized in 20 volumes of cold lysis buffer (50mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail). After a low speed spin (1,000 x g for 3 min) to remove large tissue chunks, the homogenate is centrifuged at 20,000 x g for 10 minutes at 4°C to pellet the membranes. The pellet is resuspended in fresh

buffer and centrifuged at the same speed a second time. The pellet is then resuspended into buffer (15 ml) containing 10 % sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay. On the day of the assay the membrane preparation is thawed and the pellet resuspended in final assay binding buffer.

Incubation and filtration

The filtration binding assay is carried out in 96-well plates in a final volume of 250 µL per well. To each well is added 150 µL membranes (3 - 20 µg protein for cells; 50 - 120 µg protein for tissue), 50 µL of the unlabeled compound (non-specifics) or buffer and 50 µL of radioligand solution in binding buffer. The radioligand is added at up to 8 different concentrations (e.g. 0.2 - 20 nM). The plate is incubated at 30 °C for 60 minutes with gentle agitation. The incubation is stopped by vacuum filtration onto 0.3 % PEI presoaked GF/C filters using a 96-well FilterMate™ harvester followed by four washes with ice-cold wash buffer. Filters are then dried for 30 minutes at 50°C. The filter is sealed in polyethylene, scintillation cocktail (Betaplate Scint; PerkinElmer) added and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

Data analysis

For each radioligand concentration, non-specific binding is subtracted from total binding to give specific binding. Bound CPM values are converted to fmoles per mg protein. Data is fitted using the saturation analysis non-linear curve fitting routines in Prism® (Graphpad Software Inc). The K_d (in nM) and B_{max} (fmol/mg or sites/cell) are derived from the saturation curve.

LIVE CELL ASSAY

Incubation and filtration

At room temperature, 150 µL of cells (50,000 - 150,000 cells) is added to each well, followed by 50 µL of “cold” unlabeled antibody or protein solution (for non-specifics) or buffer and 50 µL of [^{125}I]-labeled antibody or protein. The [^{125}I]-labeled antibody or protein is added at up to 8 different concentrations (e.g. 0.13 - 16 nM). The plate is incubated for 120 min with gentle agitation on an orbital shaker. The incubation is stopped by mild vacuum filtration onto GF/C filtermats (presoaked in buffer with BSA) using a 96-well FilterMate™ harvester. Washing is accomplished using an initial wash with ice-cold assay buffer followed by several washes with ice cold PBS. Filters are then dried under a stream of warm air. Scintillation cocktail (Betaplate Scint; PerkinElmer) is added to the filters and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

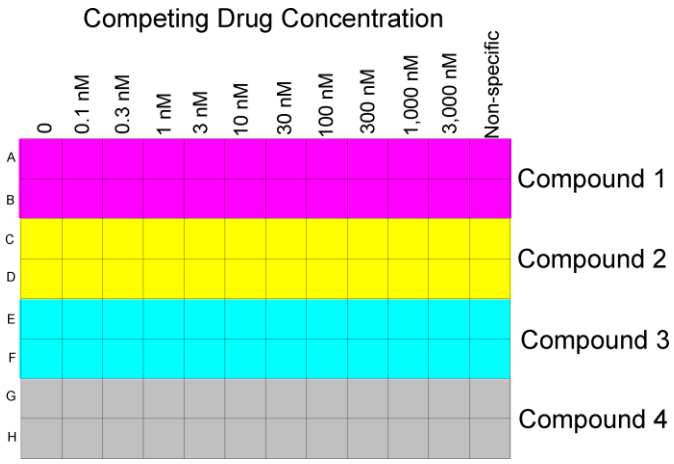
Data analysis

Bound CPM values are converted to fmoles bound [^{125}I]protein or antibody per assay well and plotted against [^{125}I]protein concentration (nM). Data is fitted using the saturation analysis non-linear curve fitting routines in Prism® (Graphpad Software Inc) to derive the K_d (in nM) and receptor or surface antigen density (B_{max}, fmol/mg or sites/cell) values.

PLATE LAYOUTS

Competition assay

Typical plate layout for 4 competing drugs with K_i values in the range of 3 - 30 nM. Duplicate replicates.



Saturation assay

Typical plate layout for 2 radiolabeled compounds, each with K_d values in the range of 0.5 - 2 nM. Triplicate replicates.