Contents

CELLULAR UPTAKE INHIBITION ASSAY 2
Cells (non-adherent) 2
Cells (adherent) 2
Synaptosomes 2
Data analysis 3
Plate layouts 3
CELLULAR RELEASE ASSAY (RUBIDIUM EFFLUX) 4
Assay protocol (adherent cells) 4
Data analysis 4

CELLULAR UPTAKE INHIBITION ASSAY

Cells (non-adherent)

The uptake assay is carried out in 96-well plates in a final volume of 250 µL per well at 37 ˚C. 150 µL of cells (50,000 – 150,000 cells) is added to each well followed by 50 µL of the competing test compound or inhibitor for defining non-specific uptake. After 30 minutes uptake is initiated by adding 50 µL of radiolabeled substrate in buffer. The plate is incubated for a predetermined interval with gentle agitation. 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 cold assay buffer followed by several washes with 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.

Cells (adherent)

Cells are seeded into 24 (or 96) well plates and incubated in full growth medium until near confluence is obtained. On the day of the assay the medium is aspirated off replaced with 0.15 ml fresh assay buffer (HBSS-HEPES, pH 7.4). To each well is added 50 µL of the competing test compound or inhibitor and the plate incubated for 30 minutes. Uptake is initiated by adding 50 µL of radiolabeled substrate in buffer. The plate is incubated for a predetermined interval with gentle agitation. The incubation is stopped by washing the cells with three changes of cold PBS. The cells are then lysed with Solvable® (50 µl). After lysis, scintillation cocktail is added to each well and the radioactivity counted in a Wallac® TriLux 1450 MicroBeta counter.

Synaptosomes

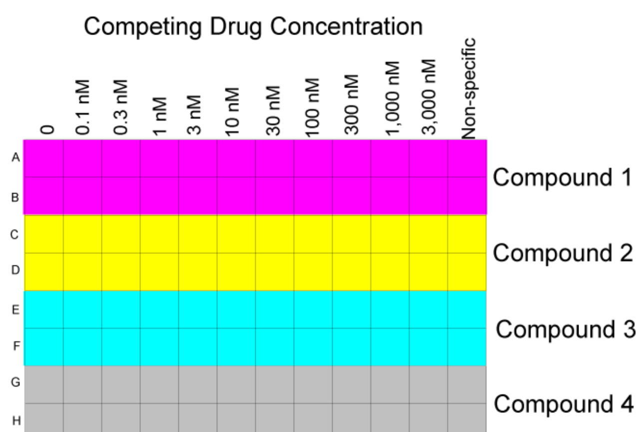
Tissue preparation: The brain is rapidly dissected from a rat, cut into pieces and placed into 20 volumes of ice-cold 0.32 M sucrose solution. The tissue is then gently homogenized in a glass homogenizer using several up-and-down strokes of the pestle. After a low speed spin (1,000 x g for 5 minutes) to remove large tissue chunks, the homogenate is centrifuged at 14,000 x g for 15 minutes at 4 °C to obtain a crude synaptosomal pellet. The synaptosomal pellet is resuspended in Krebs buffer saturated with 95% O_2 / 5% CO_2. For monoamine uptake, the buffer additionally contains ascorbate and a MAO inhibitor (pargyline). A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay.

Incubation and filtration: The uptake assay is carried out in 96-well plates in a final volume of 250 µL per well at 37 ˚C. To each well is added 180 µL synaptosomes (30 - 80 µg protein) and 50 µL of the competing test compound or inhibitor for defining non-specific uptake. After 30 minutes, uptake is initiated by adding 20 µL of ^{3}H-labeled transporter substrate in incubation buffer. The plate is incubated for a predetermined interval with gentle agitation. The incubation is stopped by gentle vacuum filtration onto GF/C filters using a 96-well FilterMate™harvester followed by three 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 (determined in the presence of a saturation concentration of a transport inhibitor) is subtracted from total binding to give specific binding. Data is fit using the non-linear curve fitting routines in Prism® (Graphpad Software Inc) to obtain the IC_{50} value for each inhibitor.

Plate layouts



Typical plate layout for testing 4 inhibitors with IC_{50} values in the range of 5 - 20 nM. Duplicate replicates.

CELLULAR RELEASE ASSAY (RUBIDIUM EFFLUX)

Assay protocol (adherent cells)

Aliquots of cells in growth medium are plated in quadruplicate onto 24-well or 96-well plates. The plated cells are grown at 37 °C for 24-48 hours to reach 70%-95% confluence. Cells are then incubated in growth medium containing $^{86}Rb^+$ (2 µCi/ml) for 4 hours at 37 °C. The labeling mixture is aspirated, and the cells are washed four times with HEPES buffer (15 mM HEPES, 140 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM Glucose, pH 7.4; 1 ml/well). Buffer, with or without agonists, is then added to each well. After incubation for 2 minutes, the assay buffer is collected. Cells are lysed by adding NaOH (0.2 M) to each well, and the lysate is then collected. The amount of $^{86}Rb^+$ in the medium and in the cell lysates is measured by liquid scintillation counting.

Data analysis

The total amount of $^{86}Rb^+$ loaded (cpm) is calculated as the sum of the assay sample and the lysate of each well. The amount of $^{86}Rb^+$ efflux is expressed as a percentage of the $^{86}Rb^+$ loaded. "Stimulated $^{86}Rb^+$ efflux" is defined as the difference between efflux in the presence of nicotinic agonists and basal efflux measured in the absence of agonists. Data is fitted using the non-linear curve fitting routines in Prism® (Graphpad Software Inc).