Supporting Assays For:

Fragment Based Discovery of Indole Inhibitors of Matrix Metalloproteinase -13

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CACO-2

Caco-2 cells were maintained at 37°C in complete Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 1.1% non-essential amino acids, 100 units/mL penicillin and 100 mg/mL streptomycin, in an atmosphere of5% CO2 and 90% relative humidity. Cells grown in 175-cm2 T-flasks were passaged every 7 days. For permeability experiments, cells were seeded at a density of 80,000 cells/cm2 in Costar 12-well plates on Transwellâ polycarbonate filters (12 mm in diameter, and with 0.4 mm pore size). The medium (0.5 mL in the insert and 1.0 mLin the well) was changed every other day for the first seven days and every day thereafter. The cells were allowed to grow and differentiate for 21 to 25 days. Culture medium was aspirated from monolayers prior to the experiment. 1x Hank's Balanced Salt Solution (pH 7.4)was added to both the inserts (0.5 mL) and the wells (1.0 mL), and allowed to equilibrate at 37°C for 30 minutes, andthen removed.A dosing solution of the test compound was prepared in 1x Hank's Balanced Salt Solution (pH 7.4) at a nominalconcentration of 50 mM. The dosing solution was then allowed to equilibrate overnight at room temperature, whilebeing mixed on a magnetic-stirrer. Prior to the experiment, the dosing solution was filtered through a 0.45 mm PVDF filter to remove any insoluble substances. The actual concentration of the dosing solution was represented by peakarea during the data analysis.For the experiment, the dosing solution was added to the donor side of the monolayers, the apical side for an A/B experiment, and the basolateral side for a B®A experiment. 1x Hank's Balanced Salt Solution (pH 7.4) was added tothe receiver side of the monolayers.At each time point, samples were collected from the receiver side of the monolayers. BothA®B and B®Aexperiments were conducted in duplicate, and samples were collected at 15, 30, 60, 90, 120 and 150 minutes. Analysis was done by LC/MS/MS, and the permeability coefficients (cm/s) were determined.

hLM (human liver microsome stability assay)

The data collected are analyzed to calculate a half-life (t1/2, min) for test compounds. The assay is performed in 50 mM potassium phosphate buffer, pH 7.4 and 2.5 mM NADPH. Test samples are dissolved in acetonitrile for a final assay concentration of 1 - 10 uM. Human liver microsomes are diluted in assay buffer to a final assay concentration of 1 mg protein/ml. A

volume of 25 ul compound solution and 50 ul microsome suspension are added to 825 ul assay buffer. The preparation is incubated for 5 min in a 37 oC water bath. The reaction is started by the addition of 100 ul NADPH. Volumes of 80 ul are removed from the incubation mix at 0 and 15 or 30 min after the start of the reaction and added to 160 ul acetonitrile. The samples are shaken for 20 sec and then centrifuged for 3 min at 3000 rpm. A 200 ul volume of the supernatant is transferred to 0.25 mm glass fiber filter plates and centrifuged for 5 min at 3000 rpm. Injection volumes of 10 ul are typically added to Zorbax SB C8 HPLC columns with formic acid in water or acetonitrile at a flow rate of 1.5 ml/min. Percent loss of parent compound is calculated from the area under each time point to determine the half-life which is then converted relative to hepatic blood flow.

rLM (rat liver microsome stability assay)

The data collected are analyzed to calculate a half-life (t1/2, min) for test compounds. The assay is performed in 50 mM potassium phosphate buffer, pH 7.4 and 2.5 mM NADPH. Test samples are dissolved in acetonitrile for a final assay concentration of 1 - 10 uM. Sprague Dawley rat liver microsomes are diluted in assay buffer to a final assay concentration of 1 mg protein/ml. A volume of 25 ul compound solution and 50 ul microsome suspension are added to 825 ul assay buffer. The preparation is incubated for 5 min in a 37 oC water bath. The reaction is started by the addition of 100 ul NADPH. Volumes of 80 ul are removed from the incubation mix at 0 and 15 or 30 min after the start of the reaction and added to 160 ul acetonitrile. The samples are shaken for 20 sec and then centrifuged for 3 min at 3000 rpm. A 200 ul volume of the supernatant is transferred to 0.25 mm glass fiber filter plates and centrifuged for 5 min at 3000 rpm. Injection volumes of 10 ul are typically added to Zorbax SB C8 HPLC columns with formic acid in water or acetonitrile at a flow rate of 1.5 ml/min. Percent loss of parent compound is calculated from the area under each time point to determine the half-life which is then converted relative to hepatic blood flow.

logP

Samples were pre-weighed (~1.0mg) into 1.2-mL conical vials. Add 1.0mL of internal stock solution (containing Toluene and Triphenylene) to each vial. Samples were sonicated for 5

minutes to dissolve the compounds and transferred directly to 1-ML amber HPLC vials. Samples were injected on HPLC with the gradient Method containing either pH 2, 7 or 10 buffer as an aqueous phase.

HPLC GRADIENT METHOD:

Column: 20*4.0 mm 5µm ODP-50 Cartridge Column with column holder

Column Temperature: 22°C

Injection Volume: 2µL

Solvents

Time (min.) Flow Rate A; B; C: Buffer D: Methanol

0.0 1.5 90 10

9.4 1.5 0 100

11.0 1.5 0 100

11.5 1.5 90 10

16.0 1.5 90 10

UV Wavelengths: 260, 285, 230, 310nm

Calculations for LogP using Retention time and internal standards: Record retention time of compound (RTcmpd) and retention time of Toluene and Triphemylene from HPLC chromatograms.

Retention Time for Toluene (internal standard):(RT1) 6.11

LogPValue for Toluene (LogP1): 2.72

Retention Time for Tryphenylene (internal standard): (RT2) 9.86

LogP Value for Tryphenylene (LogP2): 5.49

Equation:

Log P = [{(LogP1 - LogP2)* RTcmpd + (RT1*LogP2)-(RT2*LogP1)}/RT1 - RT2)]

Aq sol 7.4 (AQUEOUS SOLUBIULITY)

Preparation of Reference UV plate: 10 ul of each stock sample (including DMSO control) is added to 190 ul of propanol to prepare the reference stock plate. Reference stock samples are mixed and 5ul of each stock sample is added to UV blank plate after it is read spectrophotometrically. Sample Preparation: 5ul of each sample (including DMSO control) is added to the deep well plate containing 1000 ul of pH 7.4 buffer, mixed and incubated for 16-19 hours. The plate is sealed well during the incubation process At the end of the incubation period, 100 ul of sample from deep well plate is vacuum filtered using a filter plate. Another 200ul of the sample from deep well plate is vacuum filtered using the same filter block but a clean filter plate. 75ul of the filtrate from the filter plate is transferred to a UV sample plate. 72ul of propanol is added to this UV plate. The solution is mixed and the spectrum is read using the UV spectrophotometer. Data Analysis: The spectra collected for blank, reference and sample from 250-498nm is analyzed using pION software.

CYP2C9 Assay

Each test compound is assayed assayed in 25 mM KPO4, 1.3mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl2, and 0.4 Units/ml glucose-6-phosphate dehydrogenase. Test samples, which were previously dissolved in acetonitrile or DMSO, are separately diluted in assay buffer. Replicate 100 ml aliquots of diluted test compound are dispensed into 96-well assay plates (Packard Optiplate), and the plates are preincubated for at least 10 min at 37 C. A 100 ml volume of human recombinant YP2C9 (1.0 pmol) and substrate 7-methoxy-4-trifluoromethylcoumarin (75 mM) are then added to each test well. The reaction is incubated for 45 min at 37 oC. A standard inhibition curve using sulfaphenazole is run with each assay plate. The reaction is terminated with the addition of 75 ml 80% acetonitrile/20% 0.5 M Tris base. The plate is read with a fluorescent plate reader (Tecan Spectrafluor or LJL Biosystems Analyst) at an excitation

wavelength of 409 nm and an emission wavelength of 530 nm. The IC50 values are calculated using SAS analysis.

CYP3A4BFC Assay

Eight to ten concentrations (in half-log intervals) of each test compound are assayed assayed in 200 mM KPO4, 1.3 mM NADP+, 3.3 mM glucose-6- phosphate, 3.3 mM MgCl2, and 0.4 Units/ml glucose-6-phosphate dehydrogenase. Test samples, which were previously dissolved in acetonitrile or DMSO, are separately diluted in assay buffer. Replicate 100 ml aliquots of diluted test compound are dispensed into 96-well assay plates (Packard Optiplate), and the plates are reincubated for at least 10 min at 37 oC. A 100 ml volume of human recombinant CYP3A4 (3.0 pmol) and substrate 7- benzyloxy-4-(trifluoromethyl)-coumarin (50 mM) are then added to each test well. The reaction is incubated for 30 min at 37 oC. A standard inhibition curve using ketoconazole is run with each assay plate. The reaction is terminated with the addition of 75 ml 80% acetonitrile/20% 0.5 M Tris base. The plate is read with a fluorescent plate reader (Tecan Spectrafluor or LJL Biosystems Analyst) at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. The IC50 values are calculated using SAS analysis.

In Vivo Pharmacokinetics

Compounds were tested in Srapgue Dally Rats (N=3) by i.v. (1mpk, 70% PEG) or oral gavage as a suspension (10 mpk, methylcellulose). The compound was administered intravenously to rats and blood samples were taken at various time points post dose. The blood samples were anticoagulated and centrifuged to recover plasma which was then analyzed to quantify concentrations of parent compound. PK parameters were calculated using non compartmental methods. The compound was administered orally to rat and blood samples were taken at various time points post dose. The blood samples were anticoagulated and centrifuged to recover plasma which was then analyzed to quantify concentrations of parent compound. PK parameters were calculated using non compartmental methods.