p38α Mitogen-activated protein Kinase Inhibitors: Optimization of a series of biphenylamides, to give a molecule suitable for clinical progression

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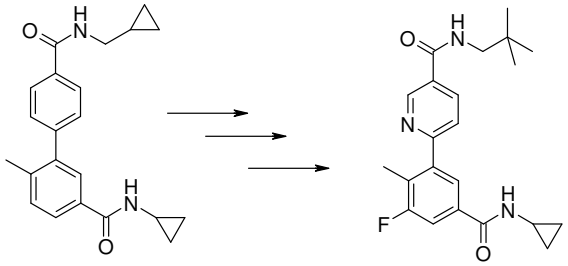
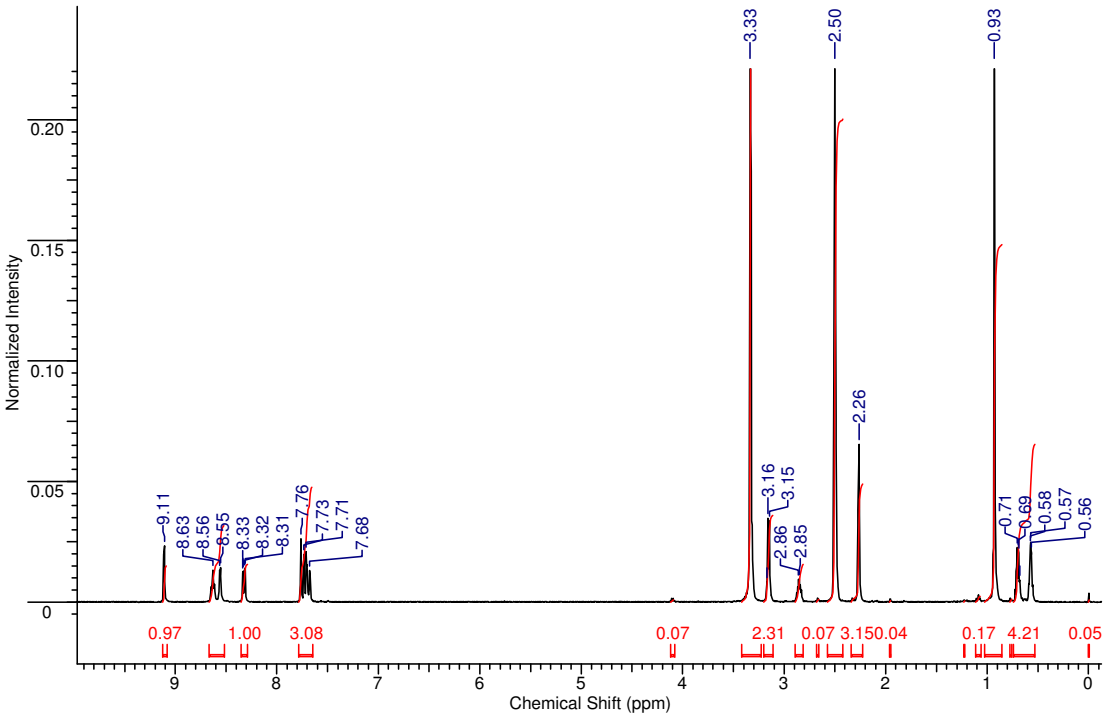


NMR Spectrum of compound 2



Cassette Rat PK. The plasma pharmacokinetics of compounds were determined in male Lewis rats after dosing a mixture of three compounds plus standard compound **1** orally at 0.25mg/kg each. The compounds were formulated as a solution (1mg/ml total) in DMSO:PEG200:water 10:70:20 (v/v/v). Blood was collected over a 24hour time period. Plasma was prepared following centrifugation and compound extracted from 50µL plasma using protein precipitation with acetonitrile. Samples were then evaporated under nitrogen and re-dissolved in 100µl of 10:90 acetonitrile: water.

Analysis was performed using LC-MSMS on the API365 Sciex mass spectrometer with a 5min fast gradient comprising 0.1% formic acid in water and 0.1% formic acid in acetonitrile (mobile phases), 20µl injection volume, flow rate 0.4ml/min and ODS3 Prodigy column (50 x 2.1mm, 5µm). Pharmacokinetic data was generated using non compartmental analysis.

Discrete Rat PK. The plasma pharmacokinetics in male Lewis rats were determined after dosing intravenously and orally at 1mg/kg. The compound was formulated as a solution (1mg/ml) in DMSO:PEG200:water 10:70:20 (v/v/v). Blood was collected over a 24hour time period. Plasma was prepared following centrifugation and compound extracted from 50µL plasma using protein precipitation with acetonitrile. Samples were then evaporated under nitrogen and re-dissolved in 100µl of 10:90 acetonitrile: water. Analysis was performed using LC-MSMS on the API365 Sciex mass spectrometer with a 5min fast gradient comprising 0.1% formic acid in water and 0.1% formic acid in acetonitrile (mobile phases), 20µl injection volume, flow rate 0.4ml/min and ODS3 Prodigy column (50 x 2.1mm, 5µm). Pharmacokinetic data was generated using non compartmental analysis.

Cynomolgus monkey PK. The pharmacokinetics of **2** were determined in three male Cynomolgus monkeys, with a cross-over study at 1.7 mg/kg intravenous and 1.4 mg/kg oral. **2** was formulated as a solution in DMSO:20% aq Cavitron 1:99 (v/v) for the intravenous arm (60minutes infusion) and in DMSO:6% aq Cavitron 1:99 (v/v) for the oral arm.

PG-PS model. Male Lewis rats, weighing 125-150g at the start of the study, were purchased from Harlan Olac (Bicester, UK). Each treatment group consisted of 8 rats housed in two cages of four, fed on standard rodent chow with free access to water.

Induction of arthritis. Under general anaesthesia, the rats were given an intra-articular injection of 5µg peptidoglycan-polysaccharide from Streptococcus pyogenes (PG-PS 100P from Lee Laboratories, Batch 147884) in a volume of 10µl sterile PBS into the right ankle joint. Fourteen or fifteen days later, the rats were injected intravenously with 200µg PG-PS in a volume of 0.5mls sterile PBS. This systemic injection of PG-PS reactivates the inflammation in the previously injected ankle joint and is designated Day 0.

Dosing schedule. The rats were dosed orally, twice daily, on days 0, 1 and 2, with 8hours between the daily doses. The first dose was given 1hour prior to reactivation of arthritis on Day 0.The dosing volume was 4mls/kg body weight. Treatments were randomized between the groups and coded so that the assessments were performed blind. Each study contained an arthritic control group dosed with vehicle 0.5% methyl cellulose in water and in most studies, a group dosed with prednisolone (2mg/kg as a positive treatment control, together with three or four dose levels of compound. The experiment was terminated on Day 3.

Assessments (Ankle swelling). The diameter of the injected (right) ankle was measured on Days 0, 1, 2 and 3 using calipers. The Day 0 measurement represents the starting diameter and was subtracted from the subsequent measurements so that the results were expressed as change in ankle diameter.

Assessments (Pain). The hypersensitivity to pain was determined by measuring weight bearing using an instrument made in-house (dual channel weight averager).

Normal rats distribute their body weight equally between the two hind paws, but when the left hind paw is inflamed and /or painful, the weight is re-distributed so that less weight is put on the affected paw (decrease in weight bearing). Assessment of this change is a sensitive method for measuring 'incident pain' and allodynia. Baseline weight bearing readings were taken on Day -14 just before sensitisation, and Day -1 (the day before the reactivation injection). After reactivation on Day 0 and the induction of inflammation and pain, the weight bearing measurements were taken on Days 1, 2 and 3 post reactivation. The results were expressed as the difference between the weight applied to the non-inflamed paw compared with the inflamed paw. This number becomes larger as the pain increases.

Data Analysis. The ankle swelling data are expressed as mean change in ankle diameter (ankle swelling) + standard error of the mean (SEM), and % inhibition of ankle swelling compared with the vehicle control, for each treatment group. Statistical analysis was carried out on the ankle swelling data collected on Day 3, when the experiment was terminated, using a Kruskal-Wallis test, and comparing each drug treated group with the vehicle treated group in individual experiments. The ED50 was calculated as the dose estimated to induce 50% inhibition compared with the vehicle control, by linear regression. For the weight-bearing assessment of pain, statistical analysis was carried out using a Kruskal-Wallis test, and comparing each drug treated group with the vehicle treated group.

Murine CIA model

Male DBA/1 mice, at the age of 10-12 weeks, were purchased from Janvier Elevage (France). Each treatment group consisted of 10 mice housed in a single cage with free access to food and water at all times.

Induction of arthritis. Arthritis was induced using a two-step immunization protocol. On Day 0, the mice were immunized subcutaneously at the base of the tail with bovine collagen type II emulsified in Freund's Complete Adjuvant. On Day 21, the mice were boosted with an intra-peritoneal injection of bovine collagen diluted in PBS.

Dosing schedule. On the first day of dosing mice showing initial signs of mild disease were divided evenly between the individual dosing groups such that each group included mice already showing symptoms with the remaining mice not yet showing symptoms. The mice were dosed orally, twice daily, on days 22 to 36 inclusive with compound in a 1% DMSO : 99% methyl cellulose (0.5% in water) vehicle. A control group was dosed with vehicle po b.i.d. as a positive control for the disease induction. The experiment was terminated on Day 36, when the following assessments were made.

Clinical score. The **c**linical score provided an estimate of inflammation. This was recorded throughout the 14 day study period at 2-3 day intervals. A sliding scale of 0 (no inflammation) to 2 (severe swelling) was applied to each of the four paws, thus the maximum score attainable per animal was 8.

Histological analysis. At the end of the study, on Day 36, the mice were killed and one knee joint removed and placed in formalin for subsequent histological analysis. Paraffin blocks were made and slides cut and stained with H&E and Safranin O. Six separate sections of each joint were examined and the following histological

characteristics were assessed and scored (blinded to the treatments): infiltrate (cellular infiltrate into the synovium), exudate (cellular migration into the joint space), chondrocyte death and proteoglycan loss (markers of cartilage damage) and bone apposition (new bone formation). Pathological changes were graded from 0 (no effect) to 3 (very marked).