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Chemistry & Drug Discovery

Supporting Information

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1. Synthesis of compound **4**:

To 101 mg of a known intermediate 2 [WO 2001019829] and 330 mg polymer-bound Triphenylphosphine (polymerlab) in 5 ml THF, 200 mg (2.0 eq.) of 3-OH N-Boc piperidine was added followed by 0.099 ml diisopropyl diazodicarboxylate. The reaction mixture stirred at room temperature overnight. After filtered off resins, the reaction mixture was concentrated and purified with flash chromatography (pentane/ethyl acetate = 1/1) to give 55 mg of intermediate 3. This compound (48.3 mg) was treated with 1 ml of 4N HCl in dioxane for 1 hour and concentrated to dryness, which was dissolved in dichloromethane and 0.042 ml of triethylamine, followed by 0.010 ml of acryl chloride. The reaction was stopped after 2 hours. The reaction mixture was washed with 5wt% citric acid (aq.) and brine, dried with MgSO4, and concentrated. Flash chromatography with (CH2Cl2/MeOH = 25/1) gave 22 mg of compound **4** as white solids. MS (M+1): 441.2; 1H-NMR (400MHz): 8.26, s, 1H; 7.65, m, 2H; 7.42, m, 2H; 7.1-7.2, m, 5H; 6.7-6.9, m, 1H; 6.1, m, 1H; 5.5-5.7, m, 1H; 4.7, m, 1H; 4.54, m, 0.5H; 4.2, m, 1H; 4.1, m, 0.5H; 3.7, m, 0.5H; 3.2, m, 1H; 3.0, m, 0.5H; 2.3, m, 1H; 2.1, m, 1H; 1.9, m, 1H; 1.6, m, 1H

2. Kinase enzymology assays:

Kinase activity was determined using a time-resolved fluorescence resonance energy transfer (TR-FRET) methodology. Measurements were performed in a reaction volume of 50 µL using 96-well assay plates. Kinase enzyme, inhibitor, ATP (at the K_m for the kinase), and 1 µM peptide substrate (Biotin-AVLESEEELYSSARQ-NH_2) were incubated in a reaction buffer composed of 20 mM Tris, 50 mM NaCl, $MgCl_2$ (5-25 mM depending on the kinase), $MnCl_2$ (0-10 mM), 1 mM DTT, 0.1 mM EDTA, 0.01% bovine serum albumin, 0.005% Tween-20, and 10% DMSO at pH 7.4 for one hour. The reaction was quenched by the addition of 1.2 equivalents of EDTA (relative to divalent cation) in 25 µL of 1x Lance buffer (Perkin-Elmer). Streptavidin-APC (Perkin-Elmer) and Eu-labeled p-Tyr100 antibody (Perkin-Elmer) in 1x Lance buffer were added in a 25 µL volume to give final concentrations of 100 nM and 2.5 nM, respectively, and the mixture was allowed to incubate for one hour. The TR-FRET signal was measured on a multimode plate reader with an excitation wavelength of 330 nm and detection wavelengths of 615 and 665 nm. Activity was determined by the ratio of the fluorescence at 665 nm to that at 615 nm. For each compound, enzyme activity was measured at various concentrations of compound. Negative control reactions were performed in the absence of inhibitor in replicates of six, and two no-enzyme controls were used to determine baseline fluorescence levels. Inhibition constants {Ki(app)} were obtained using the program BatchKi (Kuzmic et al. *Anal. Biochem.* **2000**, *286*, 45-50). IC50s were obtained according to this equation: $IC50 = \{Ki(app)/(1+[ATP]/K_m^{ATP})\} + [E]_{total}/2$; for all kinases, $[ATP] = K_m^{ATP}$, $[Btk]_{total} = 0.5$ nM and $[Lck]_{total} = 6$ nM.

3. Cellular Calcium Flux Assays:

Calcium assay was done according to manufacturer descriptions (Molecular Devices). In brief, actively growing Ramos B-cells (ATCC) in RPM1 medium supplemented with 10% FBS (Invitrogen) were washed and re-plated in low serum medium to approximately $5X10^5$ cells per 100ul per well in 96-well plate. Compounds to be assayed were dissolved in DMSO, diluted to appropriate concentrations in low serum medium (from 0 to 10uM final concentrations at dilution factor of 0.3), added to each well (the final DMSO concentration was 0.01% in each well) and incubated at 37 degree in 5% CO2 incubator for 1 hour. 100ul calcium assay dye (Calcium 3 assay kit, Molecular Devices) was then added to each well and incubated for an additional one hour. The compound treated cells were stimulated with a goat anti-human IgM antibody (80ug/ml; Jackson ImmunoResearch) and read in a Flexstation II384 (Molecular Devices) at Ex=485nm and Em=538nm for 200 seconds. The relative fluorescence unit (RFU) and the fifty percent of inhibition (IC50%) were recorded and analyzed using a built-in SoftMax program (Molecular devices).

4. Exposure levels of compound **4**:

Female Balb/c mice were treated with 100 mg/kg of Chemicon mAb cocktail to Type II collagen intravenously on Day 0 and with 1.25 mg/kg of LPS intraperitoneally on Day 1. Compound **4** was administered orally in a methylcellulose-based aqueous suspension formulation at 1, 3, 10 and 30 mg/kg once daily starting on Day 2 through Day 12. Blood samples were collected at 0.5 and 2 hours post dose of compound **4** administrations on Day 12. The serum concentrations of compound **4** were quantified by LC/MS/MS. Twenty four hour post dose, levels of compound **4** were below the level of quantitation.

Dose (mg/kg/day)	Collection Time (h)	Conc (µM)[1]	
		Mean	SD
1	0.5	0.0657	0.0153
	2	0.0485	0.0200
3	0.5	0.250	0.019
	2	0.135	0.059
10	0.5	0.635	0.053
	2	0.670	0.190
30	0.5	1.72	0.15
	2	1.10	0.19

5. Mass Spectroscopy Data for Complex of Compound **4** and BTK