Discovery of CC-99677, a selective targeted covalent MAPKAPK2 (MK2) inhibitor for autoimmune disorders

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As an anti-inflammatory strategy, MAPK–activated protein kinase–2 (MK2) inhibition can potentially avoid the clinical failures seen for direct p38 inhibitors, especially tachyphylaxis. CC-99677, a selective targeted covalent MK2 inhibitor, employs a rare chloropyrimidine that bonds to the sulfur of cysteine 140 in the ATP binding site via a nucleophilic aromatic substitutions (SNAr) mechanism. This irreversible mechanism translates biochemical potency to cells shown by potent inhibition of heat shock protein 27 (HSP27) phosphorylation in LPS-activated monocytic THP-1 cells. The cytokine inhibitory profile of CC-99677 differentiates it from known p38 inhibitors, potentially suppressing a p38 pathway inflammatory response while avoiding tachyphylaxis. Dosed orally, CC-99677 is efficacious in a rat model of ankylosing spondylitis. Single doses, 3 to 400 mg, in healthy human volunteers show linear pharmacokinetics and apparent sustained tumor necrosis factor-α inhibition, with a favorable safety profile. These results support further development of CC-99677 for autoimmune diseases like ankylosing spondylitis. (Translational Research 2022; 249:49–73)

Abbreviations: AE = adverse event; ARE = adenylate-uridylate-rich elements; AS = ankylosing spondylitis; ATF2 = activating transcription factor 2; AUC = area under the curve; BE = biochemical efficiency; C = cysteine; C140 = cysteine 140; CAMKK2 = calcium/calmodulin-dependent protein kinase 2; CD = Crohn’s disease; Cmax = peak concentration; CV = coefficient of variance; Dyrk = dual specificity tyrosine phosphorylation regulated; EC50 = half-maximal effective concentration; ERK5 = extracellular signal-regulated kinase 5; FGFR4 = fibroblast growth factor receptor 4; FIH = first in human; HSP27 = heat shock protein 27; HuR = human antigen R; IC50 = half-maximal inhibitory concentration; IS = internal standard; LC = liquid chromatography; LRRK2 = leucine-rich repeat kinase 2; MALDI = matrix-assisted laser desorption/ionization; MKP-1 = MAPK phosphatase-1; MCP-1 = monocyte chemotactic protein 1; MK2 = MAPK-activated protein kinase-2; MK2i = MK2 inhibitor; MK3 = MAPK-activated protein kinase-3; MS = mass spectrometry; MSD = Meso Scale Discovery; MSK1 = mitogen- and stress-activated protein kinase 1; MS/MS = tandem mass spectrometry; MTHD =
methyltetrahydrodiazepinone; NOMID = neonatal-onset multisystem inflammatory disease; pHSP27 = HSP27 phosphorylation; PK = pharmacokinetics; PMA = phorbol myristate acetate; PoA = prevention of activation; PRAK = p38-regulated/activated protein kinase; QC = quality control; RA = rheumatoid arthritis; SILAC = stable isotope labeling with amino acids; SMaSh = Streptavidin Mass Shift; S_nAr = nucleophilic aromatic substitutions; STK40 = serine/threonine kinase 40; TCI = targeted covalent inhibitor; Tmax = time to Cmax; TTP = tristetraprolin

At A Glance Commentary
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Background
Numerous direct inhibitors of p38 kinase have been developed for inflammatory disorders, but these have repeatedly failed. Targeting other network members, like the MAPK-activated protein kinase-2 (MK2) downstream of p38, might allow for the sustained suppression of cytokine production required for therapeutic benefit. However, no MK2 inhibitors have been clinically approved to date.

Translational Significance
We have discovered and characterized a potent, selective, covalent MK2 inhibitor, CC-99677. Orally-administered CC-99677 demonstrated efficacy in a rat model of ankylosing spondylitis and showed favorable safety in healthy human subjects. Cytokine blockade by CC-99677 may provide a potential therapy for cytokine-mediated, inflammatory disorders.

INTRODUCTION
Inflammatory cytokines are important in autoimmune, autoinflammatory, and inflammatory conditions. Cytokine neutralizing biologics have demonstrated therapeutic utility in inflammatory diseases like psoriasis,1 Crohn’s disease (CD),2 rheumatoid arthritis (RA),3 and spondyloarthritis,4 However, the need for parenteral administration and an observed loss of therapeutic benefit with time5 justify developing new orally available therapies for inflammatory diseases. In 1994, p38—MAPK was identified as a key regulator of inflammatory cytokine biosynthesis6 and was a prime small-molecule drug target, spurring the development of numerous p38 kinase inhibitors. Many p38 kinase inhibitors achieved efficacy in animal disease models7 but did not demonstrate adequate safety for further development. Three phase 2 compounds that were evaluated in patients with RA,8,9 or CD10 initially decreased disease scores or biomarkers of inflammation, but these benefits were not maintained with continued treatment. This tachyphylaxis has been attributed to repression of p38 because, in addition to initiating inflammatory responses, p38 also actuates opposing feedback mechanisms.11 Nonetheless, modulating the p38 signaling pathway still holds great potential. Targeting pathway members upstream12 or downstream11,13 of p38 might circumvent regulatory feedback and provide the sustained suppression of inflammatory cytokines needed for a durable therapeutic benefit.

MAPK-activated protein kinase-2 (MK2), a direct downstream substrate of p38, mediates the inflammatory process by increasing the translation and stability of pro-inflammatory cytokine mRNAs including IL-614 and TNFα.15 The regulation appears to be explained, in part, by the competition of 2 proteins for binding to the same adenylate-uridylate-rich elements (ARE) in the 3’ untranslated region of cytokine mRNAs (eg, TNFα). Tristetraprolin (TTP), a zinc finger protein, destabilizes such mRNAs and represses translation while human antigen R (HuR) plays an opposing role and stimulates translation. Activated MK2 phosphorylates TTP causing the latter to be displaced by HuR.16 This translation switch regulates cytokine production. MK2 is an attractive druggable target as evidenced from knockout mouse data.17 These mice appear normal; resist LPS-induced endotoxic shock, exhibiting reduced levels of TNFα, IL-6, and interferon-γ;17 and are resistant to inflammatory disease in numerous models,11 including a collagen-induced arthritis model.18 Furthermore, an ATP-competitive inhibitor demonstrated efficacy in a streptococcal cell wall-induced arthritis rat model.13 Although no evidence implicates MK2 in a complex feedback pathway, like that of p38, its activation does actuate signal feedback that could limit inflammation at the level of TTP-regulated translation or mRNA stability.16

Inhibition of MK2 can be achieved by prevention of activation (PoA) inhibitors such as ATI-450 (formerly CDD-450),19,20 which prevent MK2 activation by p38, or through orthosteric ATP-competitive mechanisms that directly inhibit the enzyme’s ability to phosphorylate all substrates. Numerous biochemically potent ATP-competitive MK2 inhibitors have been developed, but none have been approved clinically; a common problem has been poor biochemical efficiency (BE), the ratio of target affinity to cellular potency.21
Shifts to lower potency in cells of 10- to 100-fold are observed for many chemotypes. Given the high affinity of MK2 for ATP cited in the literature, 30 and 43 μM, for the inactive vs active enzyme, respectively, this potency loss has been attributed to competition with the high ATP concentration in cells, 0.5 to 5 mM.\textsuperscript{23,24} In addition, crystal structures of the active kinase conformation reveal a deep, narrow ATP-binding cleft.\textsuperscript{25} Consequently, that site accommodates planar molecules plagued by poor drug properties, like low solubility. A clinically active ATP-competitive MK2 inhibitor must overcome poor BE and have adequate exposure and selectivity against off-targets that allow the target engagement required for therapeutic utility, a need that is so far unmet. To create an MK2 inhibitor that addresses these hurdles, we turned to engineering a targeted covalent inhibitor (TCI), a strategy that has worked for addressing potency and other challenges for kinase-targeted therapies.\textsuperscript{26,27}

CC-99677 [(R)-3-((2-chloro-5-(ethoxymethyl)pyrimidine-4-yl)oxy)-10-methyl-9,10,11,12-tetrahydro-8H-[1,4]diazepino[5′,6′:4,5]thieno[3,2-f]quinolin-8-one] is a potent, selective MK2 inhibitor (MK2i). It employs an unprecedented 2-step mechanism, first reversibly binding to the ATP site then subsequently undergoing a nucleophilic aromatic substitution (SNAr) reaction between the chloropyrimidine electrophilic moiety and the cysteine 140 (C140) thiol at the kinase hinge, resulting in a covalent thioether bond. This mechanism allows for good BE as demonstrated by potent inhibition of heat shock protein 27 (HSP27) phosphorylation and several inflammatory cytokines, like TNFα, in LPS-activated monocytic THP-1 cells. CC-99677 demonstrates a cytokine profile different from that of p38 inhibitors, opening the possibility of avoiding the negative consequences of p38 inhibition and addressing inflammatory diseases in which these cytokines are active, like ankylosing spondylitis (AS). When orally dosed, this TCI is efficacious in a rat model of AS. Because CC-99677 appears fit for clinical assessment, we ran a phase 1 trial efficacious in a rat model of AS. When orally dosed, this TCI is efficacious in a rat model of AS. Because CC-99677 appears fit for clinical assessment, we ran a phase 1 trial.

### MATERIAL AND METHODS

Reagents. Compounds 1-5 (Table I) and the 4,6-substituted fluoropyrimidine amino-methyltetrahydrodiazepinone (amino-MTHD) were synthesized as described in Alexander et al.\textsuperscript{20} SCIO-469 (talmiprodil), BMS-582949, and tofacitinib (along with all other commercial reagents or tool compounds) were purchased from Sigma Aldrich (St. Louis, MO).

**Synthesis of CC-99677.** The synthetic scheme outlined in Supplemental Fig. 1 and the following procedures were used to synthesize 2 key intermediates (compounds S1-d and S1-f) that were used in the final production of CC-99677.

1. **5-(Hydroxymethyl)pyrimidine-2,4(1H,3H)-dione (S1-b).** To a 30-L reactor charged with 1H-pyrimidine-2,4-dione (compound S1-a; 2.0 kg, 17.9 mol, 1.0 equiv.) was added a solution of potassium hydroxide (0.7 kg, 23.3 mol, 1.3 equiv.). The resulting mixture was heated at 54°C for 2 days, then concentrated to a white thick mass. It was diluted with acetone (12.0 kg) and stirred at room temperature for 30 minutes; solid was precipitated. The solid was collected, washed with acetone (2.6 kg), and dried under vacuum at 45°C to afford compound S1-b (3.14 kg, quantitative yield) as a white solid, which was used for the next step without further purification. \(^1\)H nuclear magnetic resonance (NMR) (DMSO-d\textsubscript{6}, 400 MHz) \(\delta\): 7.38 (s, 1H), 4.07 (s, 2H).

2. **5-(Ethoxymethyl)pyrimidine-2,4(1H,3H)-dione (S1-c).** To a 30-L reactor charged with ethanol (15.0 kg) was added acetyl chloride (0.84 kg, 10.7 mol, 1.0 equiv.). The solution was stirred at 25°C for 30 minutes, then compound S1-b (1.5 kg, 10.6 mol, 1.0 equiv.) was added. The mixture was stirred at 80°C for 20 hours, then concentrated at 50°C to give viscous oil. The residual was cooled to 25°C and methyl tert-butyl ether (MTBE; 15.6 kg) was added. The resulting mixture was stirred at room temperature for 30 minutes then filtered. The solid was washed with MTBE (3.0 kg) and dried. This was followed by the addition of cold water (45.0 kg) to form a white solid. The resulting suspension was cooled to 0°C and methyl tert-butyl ether (MTBE; 15.6 kg) was added. The resulting mixture was stirred at room temperature for 30 minutes then filtered. The solid was washed with MTBE (3.0 kg) and dried under vacuum at 45°C to afford compound S1-c (1.8 kg, quantitative yield) as a white solid, which was used for the next step without further purification. \(^1\)H NMR (D\textsubscript{2}O, 400 MHz) \(\delta\): 7.64 (s, 1H), 4.25 (s, 2H), 3.60 (q, \(J=7.2\) Hz, 2H), 1.19 (t, \(J=7.2\) Hz, 3H).

3. **2,4-Dichloro-5-(ethoxymethyl)pyrimidine** (S1-d). To a 10-L reactor charged with toluene (3.1 kg) was added compound S1-c (1.8 kg, 10.6 mol, 1.0 equiv.). This suspension was cooled to 0°C–5°C under nitrogen and phosphorus oxychloride (4.05 kg, 26.4 mol, 2.5 equiv.) was added dropwise. The mixture was stirred at the same temperature for 15 minutes then N,N-diisopropylethylamine (2.03 kg, 15.7 mol, 1.5 equiv.) was added slowly while maintaining the temperature below 10°C. The resulting reaction mixture was stirred at 120°C for 2 hours. After cooled to room temperature, the mixture was poured into a stirred bi-phasic mixture of ethyl acetate and water (10 kg/10 kg) at 0°C over a period of 3 hours.
Table I.
Structure activity relationships for select MK2 inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>R*</th>
<th>Mass modification (%)</th>
<th>MK2 IC\textsubscript{50} (nM)</th>
<th>pHSP27 EC\textsubscript{50} (nM)</th>
<th>Solubility pH 2.0/7.4 (\textmu g/mL)</th>
<th>CACO-2 efflux ratio</th>
<th>Mouse 59 30 min (% remaining)</th>
<th>Biochemical efficiency (IC\textsubscript{50}/EC\textsubscript{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cl</td>
<td>0 (1)</td>
<td>35 (1)</td>
<td>1350 (1)</td>
<td>13/\textless 1</td>
<td>5/59</td>
<td>12</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>Cl</td>
<td>85 (3)</td>
<td>4.7 (2)</td>
<td>8.7 (3)</td>
<td>&lt;1/&lt;1</td>
<td>0.11/7.5</td>
<td>66</td>
<td>57</td>
</tr>
<tr>
<td>3</td>
<td>Cl</td>
<td>63 (3)</td>
<td>53.9 (4)</td>
<td>104 (3)</td>
<td>54/59</td>
<td>13/38</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>Cl</td>
<td>103 (2)</td>
<td>48.9 (2)</td>
<td>8.9 (2)</td>
<td>&gt;100/&gt;100</td>
<td>2/37</td>
<td>16</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>Cl</td>
<td>56 (3)</td>
<td>115.4 \pm 4.1 (3)</td>
<td>610 \pm 249 (4)</td>
<td>86/83</td>
<td>0.6/38.8</td>
<td>64</td>
<td>89</td>
</tr>
<tr>
<td>CC-99677</td>
<td></td>
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</tbody>
</table>

Abbreviations: EC\textsubscript{50}, half-maximal effective concentration; IC\textsubscript{50}, half-maximal inhibitory concentration.

*R represents variations of the core molecule shown at the top of the table.

^yNumbers in parentheses denote the number of independent experiments per analysis.

^zBiochemical efficiency typically uses K\textsubscript{i} as the measure of biochemical affinity. In this table, biochemical efficiency is the ratio of the biochemical IC\textsubscript{50} to the cellular EC\textsubscript{50} for phosphorylation of HSP27. We use IC\textsubscript{50} as a measure of biochemical potency because it, like the cell assay, incorporates the time-dependent nature of an irreversible mechanism, as elaborated further in the text, whereas the conventional K\textsubscript{i} (or even K\textsubscript{d}), appropriate for reversible binders, does not. IC\textsubscript{50} is defined by Eq. 1 and EC\textsubscript{50} is similarly defined for cellular inhibition measurements.
period of 1 hour to keep the temperature below 50°C. The mixture was stirred for 15 minutes then separated. The aqueous phase was extracted with ethyl acetate (4.0 kg x 2). The organics were combined, washed with brine (5%, 10 kg x 2), dried over anhydrous sodium sulfate and concentrated. The residue was purified by flash column chromatography (petroleum ether/acetate, 20/1) to afford compound S1-d (1.0 kg, 45.7%) as a yellow solid. \(^1\)HNMR (CDCl\(_3\), 400 MHz) \(\delta\) 8.67 (s, 1H), 4.57 (s, 2H), 3.67 (q, \(J = 7.2\) Hz, 2H), 1.30 (t, \(J = 7.2\) Hz, 3H).

(R)-3-Hydroxy-10-methyl-9,10,11,12-tetrahydro-8H-[1,4]diazepino[5',6':4,5]thieno[3,2-f]quinolin-8-one (S1-f). To a 5-L reactor charged with glacial acetic acid (7.5 kg) and water (2.5 kg) was added compound S1-e (0.95 kg, 2.99 mol), prepared as described in Anderson et al.\(^{29}\) The mixture was stirred at 110°C for 4 days then cooled and concentrated to remove most of the solvents. Ammonium hydroxide (6.0 kg) was added to the residue and the resulting mixture was stirred at room temperature for 1 hour then filtered. The crude product was slurried with ethyl acetate (10 kg) for 1 hour then filtered. The solid was washed with ethyl acetate (2 kg), dried under vacuum at 55°C to afford compound S1-f as a yellow solid (0.78 kg, 87.6%). \(^1\)HNMR (DMSO, 400 MHz) \(\delta\) 11.99 (s, br, 1H), 8.01 (d, \(J = 10.0\) Hz, 1H), 8.12 (d, \(J = 4.0\) Hz, 1H), 7.94 (d, \(J = 8.8\) Hz, 1H), 7.44 (d, \(J = 8.8\) Hz, 1H), 6.88 (t, \(J = 4.8\) Hz, 1H), 6.01 (d, \(J = 10.0\) Hz, 1H), 3.50-3.60 (m, 1H), 3.35-3.45 (m, 2H), 1.16 (d, \(J = 6.8\) Hz, 1H).

(R)-3-(2-Chloro-5-(ethoxymethyl)pyrimidin-4-yl)oxy)-10-methyl-9,10,11,12-tetrahydro-8H-[1,4]diazepino[5',6':4,5]thieno[3,2-f]quinolinelin-8-one (CC-99677). To a 20-L reactor charged with dimethyl sulfoxide (9.2 kg) was added compound S1-f (0.4 kg, 1.34 mol, 1.0 equiv.) and potassium carbonate (0.39 kg, 2.83 mmol, 2.0 equiv.). The mixture was stirred at 20°C-25°C for 40 minutes, then compound S1-d (0.44 kg, 2.13 mol, 1.5 equiv.) was added. The reaction was stirred at 20°C-30°C for 36 hours, then quenched by addition of water (9.0 kg). The resulting mixture was stirred for 30 minutes and filtered. The solid was washed with water (9.0 kg) and dried at 45°C under vacuum. The crude product was stirred at 25°C in dichloromethane (8.0 kg) for 16 hours, then filtered. The solid was washed with dichloromethane (4.0 kg) and dried at 45°C under vacuum to afford CC-99677 (0.4 kg, 63%) as a yellow solid. \(^1\)HNMR (DMSO-d6, 400 MHz) \(\delta\) 9.37 (d, \(J = 8.8\) Hz, 1H), 8.72 (s, 1H), 8.19 (d, \(J = 8.8\) Hz, 1H), 8.16-8.17 (m, 1H), 7.86 (d, \(J = 8.8\) Hz, 1H), 7.64 (d, \(J = 8.8\) Hz, 1H), 7.18 (t, \(J = 5.2\) Hz, 1H), 4.66 (s, 2H), 3.64 (t, \(J = 6.8\) Hz, 1H), 3.61-3.63 (m, 1H), 3.46-3.50 (m, 2H), 2.55 (s, 2H), 1.18-1.23 (m, 6H). Liquid chromatography with mass spectrometry (LC/MS): C\(_{22}\)H\(_{20}\)Cl\(_5\)N\(_5\)O\(_3\)S Calc. 469.94; Found 470.3 (M+H).

High-performance liquid chromatography (HPLC)—210 nm, \(rt = 11.41\) min (99.44%); 254 nm, \(rt = 11.41\) min (99.70%). HPLC conditions: Column: Agilent zorbax SB-C8, 3.5 μm, 150 mm(L) x 4.6 mm (ID)Flow Rate 1.0 ml/min, Mobile phase A: H\(_2\)O/H\(_2\)CO\(_2\)H (1000/0.25); Mobile phase B: CH\(_3\)CN/H\(_2\)CO\(_2\)H (1000/0.25); LC, MS, and \(^1\)HNMR spectra of CC-99677 are shown in Supplemental Fig. 2.

**Crystallization.** The crystallization construct comprises the vector-derived residues Gly-Pro followed by residues His47 to Arg364 of MAPKAP2. The loop comprising residues Ser216-Pro237 was deleted and replaced with a linker comprising a single Gly residue. The crystals used for determining the structure MAPKAP2 kinase domain complexed with the amino-MTHD were obtained by co-crystallizing MAPKAP2 with ADP, and then soaking these crystals with the amino-MTHD. MAPKAP2 was at a concentration of 16.3 mg/mL in 200 mM NaCl, 20 mM Hepes-NaOH pH 7.5, 5 mM MgCl\(_2\), 3 mM TCEP was incubated with 5 mM ADP and 5 mM MgCl\(_2\) for 1 hour. The protein was then crystallized by hanging-drop vapor-diffusion at 20°C. 1.0 μL of the protein sample was mixed with 1.5 μL of the crystallization solution (2.8 M sodium acetate pH 7.0) and equilibrated over a reservoir containing 0.4 mL of crystallization solution. Well diffracting crystals appeared within 1 day. These were transferred to the soaking solution (4.8 mM CC073664 [diluted from a 150 mM stock solution in 100% v/v DMSO] and 3.0 M sodium acetate pH 7.0). After 24 hours the crystals were directly cooled in liquid nitrogen without the addition of any further cryo-protectant. Data were collected at beamline ID29 of the European Synchrotron Radiation Facility (Grenoble, France). Crystal data and refinement statistics for the amino-MTHD co-complex are shown in Supplemental Table 1.

**Structure determination.** Diffraction data were integrated and scaled with iMosflm\(^{30}\) and Aimless,\(^{31}\) respectively, through the CCP4i interface.\(^2\) The structure was determined by rigid-body refinement with Refmac5,\(^{33}\) using an unpublished isomorphous model of MAPKAPK2 (without any ligands) as a starting model. The model was improved through manual rebuilding of the model in Coot\(^{34}\) and restrained refinement with Refmac5. Atomic displacement factors were modeled with an isotropic B-factor per atom. The backbone geometry was analyzed with MolProbity.\(^{35}\) The restraints for the modelled compounds were generated with Libcheck.
Permeability determination. Caco-2 cells (clone C2BBe1) were obtained from American Type Culture Collection (Manassas, VA), and cell monolayers were grown to confluence on collagen-coated, microporous, polycarbonate membranes in 12-well Costar Transwell plates. Hanks Balanced Salt Solution containing 10 mM HEPES and 15 mM glucose at a pH of 7.4 was used as media buffer, 1% bovine serum albumin was added to the receiver chamber. Test article was dosed on either the apical side (A-to-B) or the basolateral side (B-to-A) at concentration of 3 μM and incubated at 37°C with 5% CO2 in a humidified incubator. Samples were taken from both donor and receiver chambers after 2 hours. The flux of co-dosed lucifer yellow was also measured for each well to confirm no damage was inflicted to the cell monolayers during the flux period. All samples were analyzed by liquid chromatography with tandem MS (LC-MS/MS) using electrospray ionization.

Determination of microsomal stability (S9). The stability of CC-99677 in mouse S9 was conducted at a starting concentration of 3 μM in the 100-mM phosphate buffer (pH 7.4; Sigma catalog # P3619), containing 0.5 mg/mL CD-1 mouse S9 (BD Biosciences, San Jose, CA, catalog # 452791), 1.0 mM nicotinamide adenine dinucleotide phosphate (NADPH; Sigma catalog # N1630) and 2.5 mM of glutathione (GSH; Sigma catalog # 6529). Samples were incubated in triplicate under 37°C for 30 minutes; aliquots of each sample were taken at both 0 and 30 minutes and quenched by adding 4× volume of acetonitrile, containing 100 nM carbamate (Sigma catalog # S385433) as internal standard (IS). The quenched samples were then filtered through a 96-well filter plate (EMD Millipore catalog # HS640) and the supernatant was analyzed using LC-MS/MS (API 4000 Triple quadruple Mass Spectrometer; Applied Biosystems). Percentage remaining of parent compound at 30 minutes was calculated as follows:

\[ \text{Percentage remaining} = \left( \frac{\text{Ratio of parent compound peak area to IS peak area at 30 minutes}}{\text{Ratio of parent compound peak area to IS peak area at 0 minutes}} \right) \times 100\% \]

Determination of MK2 covalent modification by CC-99677. Recombinant human MK2 was incubated with 10 molar equivalents of CC-99677 for 1 hour at room temperature. Samples were then prepared for MALDI analysis (AB Scinx MALDI TOF/TOF 4800 mass spectrometer with an HM2 detector; settings HV1: 2.7 kV, HV2: 20 kV, 20 ns bin size, and the laser intensity set to a constant 6000 units), where the centroid mass of the compound-treated sample was compared to the centroid mass of the untreated recombinant protein to determine the extent of modification. That number was then divided by the molecular weight of the modifying mass of CC-99677 (435 Da) and then multiplied by 100 to give the reported percent modification. Chlorine and hydrogen are eliminated upon covalent modification of MK2 by CC-99677 (MW = 469.94); therefore, the modifying mass used is the compound molecular weight minus 36.45 Da, the sum of both atoms. Samples were digested with trypsin (1:10 protease:protein ratio incubated overnight at 37°C) and analyzed via MS to identify the amino acid residue covalently modified by each compound. The mass spectra obtained from the peptide analysis was parsed manually to find differences between control and treated samples as well as to find the expected mass of the peptide containing the suspected amino acid target modified with the modifying mass of CC-99677. When differences were detected, or the expected mass was observed, that particular ion was selected for MS/MS analysis. The MS/MS spectra were manually sequenced and validated against the theoretical modified peptide ions obtained from Protein Prospector computer software that generates user-specified theoretical ions (http://prospector.ucsf.edu). Confirmation of a modified residue occurs with almost complete ion labeling of actual spectra.

Biochemical assays. The Omnia® continuous read fluorescence assay was performed essentially as described by the vendor (Invitrogen/Thermo Fisher, Carlsbad, CA) for all biochemical potency determinations. The assay employs an unnatural amino acid chemically enhanced fluorophore (Sox; 8-hydroxy-5-(N,N-dimethylsulfonamido)-2 methylquinoline) incorporated into a kinase-specific peptide substrate ST3.10 Enzyme activity was measured using activated MK2 from Invitrogen at 0.5 nM and substrate peptide and ATP at 10 μM at varying concentrations of inhibitor. The fluorescence was continually monitored at λ<sub>ex</sub>360/λ<sub>em</sub>485 every 30 seconds for 2 hours in 96-well format using a Synergy 2 plate reader (BioTek, Winooski, VT) at room temperature.

IC<sub>50</sub> determinations. Background signals from the no enzyme control wells were subtracted from all progress curves. The initial linear portions of the net progress curves were fit to a linear equation to yield the slope. This slope was used to calculate the percentage of inhibition (% Inhibition) at each compound concentration by subtracting the ratio of the slope at each inhibitor concentration to the no inhibitor (DMSO only) reference control from 1.00 and multiplying by 100. IC<sub>50</sub> and Hill slope values were determined by fitting plots of % Inhibition vs inhibitor concentration [I] according to a dose-response equation (Eq. 1) using...
GraphPad PRISM software (Version 6.00; GraphPad San Diego, CA).

\[
\% \text{ Inhibition} = \frac{100}{1 + \left(\frac{[I]}{K_i}\right)^n}
\]  

(1)

**Kinetics: determination of** $k_{\text{inact}}$ **and** $K_i$. Background signals from the no enzyme control wells were subtracted from all progress curves. The net progress curves obtained during the first 25 minutes of reactions were fit according to an ascending single exponential equation (Eq. 2) to yield $k_{\text{obs}}$ values at each compound concentration.

\[
F = V_0 \frac{1 - e^{-k_{\text{obs}}t}}{k_{\text{obs}}}
\]  

(2)

where $F$ is the fluorescence intensity from the plate reader, $V_0$ is a constant reflecting the relationship between the instrument readout and product concentration, $t$ is time, $e$ is Euler’s number, and $k_{\text{obs}}$ is the observed inactivation rate constant. Plots of $k_{\text{obs}}$ vs inhibitor concentration were fit according to Eq. 3 to generate the $k_{\text{inact}}$ and $K_i$-values using GraphPad PRISM software (Version 6.00; GraphPad; San Diego, CA).

\[
k_{\text{obs}} = \frac{k_{\text{inact}}}{2K_i + [I]} \]  

(3)

where $k_{\text{obs}}$ is the observed inactivation rate constant, $k_{\text{inact}}$ is the apparent inactivation rate constant, $K_i$ is the apparent reversible inhibitor dissociation constant, and $[I]$ is the inhibitor concentration.

**Data analysis.** In vitro binding assay analysis for safety panel. Binding of 10 μM CC-99677 to 80 receptors was performed at Eurofins using radioisotopically labeled ligands for each receptor in a competition study. The results are expressed as a percentage of control specific binding: (measured specific binding/control specific binding) × 100, and as a percentage inhibition of control specific binding: 100−[(measured specific binding/control specific binding) × 100] obtained in the presence of CC-99677.

**Kinase profiling.** The Miniaturized HotSpot℠ assay, a plate-based radiometric assay using $^{33}$P-ATP, was used at Reaction Biology Corporation to determine the percent inhibition of 1 μM CC-99677 against a panel of 364 kinases and to establish the IC$_{50}$ values for kinases showing greater than 80% inhibition. The assay directly measures kinase catalytic activity via the incorporation of $^{33}$P into a specific kinase substrate from $^{33}$P-ATP. Briefly, to substrate peptide in reaction buffer (20 mM HEPES pH 7.5; 10 mM magnesium chloride [MgCl$_2$]; 1 mM ethylene glycol-bis(2-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid [EGTA]; 0.02% Brij-35; 0.02 mg/mL Bovine Serum Albumin; 0.1 mM sodium orthovanadate [Na$_3$VO$_4$]; 2 mM dithiothreitol [DTT], 1% DMSO) kinase was added and gently mixed. Compound in 100% DMSO was then delivered using acoustic dispensing and incubated for 20 minutes at RT. The reaction was initiated by adding a mixture of ATP and $^{33}$P-ATP to a final concentration equal to the Km for ATP for each kinase. The reaction was incubated 2 hours and spotted onto P81 Whatman ion exchange filter paper. Unbound phosphate was removed by extensive washing in 0.75% phosphoric acid and then subjected to scintillation counting. Raw scintillation counts were converted to percentage of activity based on a value of 100% activity for DMSO controls and duplicates were averaged (% activity = 100 × (C$_{\text{compound}}$/C$_{\text{DMSO}}$)). IC$_{50}$ values were determined by performing a dose response to inhibitor and fitting plots of % inhibition to Eq. 1.

**Cellular assays and analysis.** Cellular activity was assessed in the THP-1 (ATCC, TIB-202) human monocytic cell line after using PMA to induce macrophage-like differentiation. After a 1-hour incubation with compound cells were stimulated with LPS to activate the MK2 signaling cascade. Inhibition of MK2 was then monitored after lysis by inhibition of HSP27 phosphorylation on Ser78, an immediate downstream target of MK2 phosphorylation, and by employing SMaSh a new assay to assess covalent target occupancy.

**Inhibition of HSP27 phosphorylation.** THP-1 cells (ATCC no TIB-202, Manassas, VA) were grown in 12-well plates at 1×10$^6$ cells/well in 1 mL of complete RPMI 1640 medium (10% FBS, 1% P/S, and 0.05 mM 2-mercaptoethanol, Invitrogen, Carlsbad, CA) and supplemented with 10 ng/mL PMA and allowed to differentiate for 3 days at 37˚C/5% CO$_2$. Cells were washed once in complete RPMI media. CC-99677 was diluted in DMSO and added to the cells to achieve a final concentration of 0 (DMSO control), 0.2, 0.5, 1.4, 4.1, 12.3, 37, 111, 333, 1000, and 3000 nM in 1 mL of complete media. After incubating for 1 hour at 37˚C, cells were washed twice in complete media and then stimulated with or without (DMSO unstimulated control) 50 ng/mL LPS for 45 minutes at 37˚C in 1 mL of complete RPMI medium. Cells were washed with PBS and then lysed in 50 μL of MSD lysis buffer supplemented with Complete Protease Inhibitor (1 tablet/10 mL), PhosSTOP (1 tablet/10 mL), and 0.5 mM phenylmethlysulfonyl fluoride (Sigma). Plates were stored at −80˚C. Plates were thawed and lysates were collected in tubes and spun at 13,000 rpm for 10 minutes. Cleared lysates (25 μL) were analyzed by Phospho (Ser78)/Total HSP27 Assay Whole Cell Lysate MSD.
Kit per manufacturer’s instructions. The remainder of the cleared lysates was used for MK2 occupancy analysis by SMaSh.

**Details of SMaSh assay.** SMaSh employs a covalent tool molecule derivatized with biotin which is added after cells are treated with CC-99677 and lysed. The details of the SMaSh assay are described fully in Labenski et al. The tool binds to unoccupied MK2 and is then exposed to excess streptavidin. Subsequent electrophoresis and Western analysis yields bands corresponding to CC-99677 occupied and unoccupied MK2 bands; the latter is a complex of MK2, tool molecule, and streptavidin and is shifted by \( \sim 55 \text{kDa} \). These bands can be visualized by 1 MK2 antibody and quantified by Western analysis on a ProteinSimple instrument to provide an estimation of occupancy.

The protein concentration of cleared lysates from cells treated with CC-99677 (vide supra) was determined by bicinchoninic acid protein quantification and then diluted in Meso Scale Discovery (MSD) lysis buffer to a final protein concentration of 1 mg/mL. An anti-MK2 primary antibody (CST12155, Cell Signaling Technology, Danvers, MA) was used at 1:50 dilution. Peggy Sue software (compass) quantifies the AUC for each peak (MK2-CC-99677 and MK2-SA complex, respectively). The percent free (% free) MK2 was determined by:

\[
\text{% Free MK2} = 100 \times \frac{(\text{Area MK2-SA})}{(\text{Area MK2-SA} + \text{MK2-CC-99677})}
\]

**Attributes and evidence used in qualifying the MK2 tool compound (CC-292TC) for SMaSh.**

1. **Binds in the ATP site:** The tetracyclic scaffold is a derivative of a known tetracyclic pyrrole series previously demonstrated to inhibit MK2. The tool compound exhibits IC\(_{50}\) ~ 80 nM, comparable to molecules in that series.

2. **Binds covalently:** The acrylamide electrophile is positioned to reach C140 when the tetracyclic scaffold is bound in the ATP site. When incubated with recombinant protein for 1 hour, MALDI analysis shows a shift of the centroid of mass of MK2 by 963 Da, corresponding to a 96% modification of MK2 confirming that this tool is covalent.

3. **Biotin moiety is attached and tethered to the aryl acrylamide electrophile with a vector designed to be solution accessible.** The tool structure is shown in Supplemental Fig. 3.

**MK2 resynthesis rate.** The rate at which MK2 turns over (t\(_{1/2}\)) in PMA-treated THP-1 cells was determined by pulse-chase SILAC and by employing the SMaSh assay.

MK2 was immunoprecipitated with the primary MK2 antibody (1:100 dilution) overnight at 4°C on a shaker. To each sample, 50 µL of protein G magnetic beads were added for 1 hour at 4°C on the shaker. Samples were then loaded on Miltenyi columns that were pre-rinsed with 100 µL of diluted RIPA lysis buffer using a MultiMACSTM. Columns were rinsed twice with 400 µL diluted RIPA lysis buffer and then with 1 mL of 50 mM Tris (pH 8.0) over 3 washes of 400, 400, and 200 µL. Columns were then removed from MultiMACSTM and MK2 samples were eluted with 100 µL of 8 M urea/TCEP (10 mM) solution. To ensure complete elution, columns were further rinsed with 50 µL of Tris (pH 8.0). The elution step was performed for 1 hour at room temperature in the dark on the shaker. Eluted samples were then incubated with 15 mM iodoacetamide for 1 hour at room temperature in the dark on shaker. Samples were then further diluted with 50 mM Tris (pH 8.0) to 500 µL to bring the urea concentration under 2 M. Peptides were generated by trypsin digestion O/N at room temperature in the dark on shaker. The protein G beads were removed from the samples by running the trypsin-digested samples through fresh Miltenyi columns using a MultiMACSTM. The beads were retained on the magnet while peptides passed through and were collected into a 500-µL Lo-bind plate. The C18 plate was then
prepared by rinsing once with 400 μL of 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) under low vacuum, followed by 2 additional rinses with 400 μL of 0.1% TFA. Peptides were then loaded onto the pre-rinsed C18 plate and then washed 3 times with 400 μL of 0.1% TFA under low vacuum. Peptides were then eluted with 400 μL of 80% acetonitrile in 0.1% TFA under low vacuum and collected in a fresh 500-μL Lo-bind plate. Peptides were then divided into 2 aliquots of equal volume in 1.5-mL Lo-bind Eppendorf tubes using Lo-bind tips. Peptides were lyophilized for approximately 4 hours under continuous heat of 70˚C and normal vacuum. Lyophilized peptides were then frozen at −80˚C until injection for LC-MS analysis. Lyophilized peptides were then resuspended in 10 μL of 2% acetonitrile with 0.1% formic acid. The entire volume was then transferred to Supelco 0.3 mL QSertVials.

Ten percent of the sample was injected into a Mass Spectrometer Quantiva™ for LC-MS analysis. Heavy to light SILAC ratios were calculated for individual MK2 peptides at each time point. The heavy and light SILAC ratios were then used to calculate resynthesis rate. The half-life value was calculated using a linear regression model to fit the logarithm of SILAC ratios.

MK2 resynthesis rate by SMaSh/prolonged duration of CC-99677. THP-1 cells, differentiated with PMA or left undifferentiated, were treated with 1 μM CC-99677 or DMSO for 1 hour at 37˚C. Cells were washed extensively to remove excess free compound. A portion of the cells was immediately lysed and served as the 0-hour timepoint. The remaining cells were grown in complete FBS media for the indicated time and stimulated with LPS (50 ng/mL) for 45 minutes prior to harvesting cells for analysis. Percentage free MK2 was determined by SMaSh and all signals were normalized to DMSO control at the specified time points. Human PBMCs, monocytes, and naive CD4+ T cells were isolated and similarly analyzed for occupancy as well.

The activation steps for these cell types were: staphylococcal enterotoxin B (1 pg/mL) + interleukin-2 (50 ng/mL); macrophage colony stimulating factor (5 ng/mL) + interleukin-3 (0.4 ng/mL) overnight then restimulation until harvested for a time point; and anti-CD3 antibody (2 mg/mL) + anti-CD28 antibody (2 mg/mL), respectively. PBMCs were isolated from 2 HLA-β27 rats and treated with 1 μM CC-99677 or DMSO for 1 hour at 37˚C. Cells were washed twice with media, lysed, and collected at 0, 6, 16, 24, 48, 72, and 96 hours post treatment.

Cytokine measurement. PBMCs were isolated from fresh whole blood from Research Blood Components, Brighton, MA. Diluted PBMCs (650 μL) were treated with 2 p38 inhibitors (SCIO-469 and BMS-582949), tofacitinib, and CC-99677 at concentrations from 4 to 3000 nM for 1 hour at 37˚C. Cells were stimulated with LPS at 50 ng/mL and then incubated for 48 hours at 37˚C at 5% CO2. After incubation, cells were centrifuged at 1200 rpm for 4 minutes and supernatants were carefully collected and cytokines were measured using Luminex. The plates were read on a Bio-Plex reader following standard manufacturer’s instructions using 50 beads/region, 60-second timeout, and 5000 to 25,000 doublet discriminator gate as read parameters. The data were analyzed using Bio-Plex Manager version 6 and the cytokines values for each analyte for all the samples were derived. These values were plotted on GraphPad PRISM software version 5 (San Diego, CA) to obtain EC50 values.

Rat model of AS and paw swelling. For a model of AS we used male HLA-B27/Huβ2m transgenic rats, which express multiple copies of the MHC I allele, HLA B27, an allele that is associated with AS in humans. Biologic agents that have demonstrated disease-modifying activity in human clinical trials (anti-TNFα and anti-IL-17α) have also shown activity in this rat model of spondyloarthritis. The JAK inhibitor, tofacitinib, has also been described to have clinical activity in human AS and served as a comparator. Doses of CC-99677 were selected to achieve submaximal and maximal MK2 target occupancy. Male rats (HLA-B27/Huβ2m) from the University of Texas South West (Dallas, TX) were orchicetomized 2 weeks prior to study start. On Day 1, 45 animals were injected with 100 μL of CFA 50 μg subcutaneously at the tail base. Two animals were not dosed as naive controls for paw swelling.

Five days following CFA injection, rats were randomized into 5 groups (n = 9/group) based upon age and parentage, and dosed with vehicle, 20 or 100 mg/kg CC-99677 or 10 mg/kg tofacitinib daily for 35 days (a biologic agent was included in the study design). Because CC-99677 solubility was low, ~1 μg/mL at pH 6.8 (phosphate buffer), formulation was critical. To conduct in vivo studies, a spray dried dispersion, composed of 35% CC-99677 and 65% hydroxypropyl methyl cellulose acetate succinate-M, was used to formulate for oral administration. The latter also performed well for in vivo preclinical and clinical studies, infra vide. Tofacitinib citrate powder was formulated as a clear solution in 0.5% methyl cellulose/0.25% Tween 80. Animals were administered CC-99677 or tofacitinib by oral gavage at a dose volume of 10 mL/kg. Vehicle was dosed to rats in the control group at the same dose volume. Control rats were left untreated. A clinical arthritis score was given to each animal 3 times per week. Hind paw volume was also measured by a plethysmometer twice weekly,
starting on Day 5. The AUC of the clinical paw swelling scores was calculated for each animal over the study time course. Differences between treatment groups were analyzed by ANOVA with Kruskal Wallis test. Percent inhibition of paw swelling was determined by comparing the AUC of each treated group to the vehicle group. On Day 40, a final dose of CC-99677 or tofacitinib was administered, and plasma was collected for PK analyses. At 24 hours post the last dose, 3 animals each in the vehicle and the CC-99677 groups were sacrificed and whole blood (for PBMCs) and spleens were collected to assess target occupancy by SMaSh.

**Preclinical pharmacokinetic sampling and analysis.**

*Pharmacokinetics in HLA-b27 rats.* Following the last dose on day 40 of running the AS rat model, approximately 200 µL of blood was collected by conscious jugular stick from animals administered CC-99677 at 0.5, 1, 2, 4, and 8 hours post dose. At 24 hours post dose, the animals were sacrificed by CO2 inhalation and approximately 1 mL of blood was collected from each rat via cardiac puncture. Blood was collected into K2EDTA tubes for plasma analysis and immediately placed on ice. Samples were centrifuged at 8000 rpm for 8 minutes. Plasma was transferred onto 96 well plates on dry ice, then stored at −80°C prior to analysis. Plasma samples were analyzed using LC-MS/MS. Individual and mean plasma concentrations at each time point were determined (n = 3 animals/gender/group). Composite PK parameters were calculated using Phoenix WinNonlin 6.3 (Certara Inc., St. Louis, MO). Peak concentration (Cmax) and time to Cmax (Tmax) were determined from actual data. AUC from 0 to time of the last detectable concentration (AUClast) was determined by the non-compartmental model using the linear trapezoidal linear interpolation rule. Plasma concentrations below the limit of quantitation were not used for AUC calculations.

*Analytical methods for CC-99677 in rat plasma.* Whole blood from the HLA-b27 rats dosed with CC-99677 in the AS disease model was collected in microtubes containing EDTA at desired time points. The tubes were centrifuged at 8000 rpm for 8 minutes, and the resulting plasma samples were taken and stored at −80°C before analysis. Plasma standard curves were prepared by adding the test compound into rat plasma and serial diluting to desired concentration. An aliquot of 50 µL of each plasma sample and each standard were added to 200 µL of acetonitrile with 100 ng/mL of carbutamide (Sigma Aldrich, St. Louis, MO), IS, for protein precipitation, then filtered through a 96-well Orochem filtration plate (Orochem Technologies Inc., Naperville, IL). The extracted test compound in resultant supernatant was analyzed with appropriate liquid chromatography column eluting to a Sciex QTRAP 6500+ LC-MS/MS system (Applied Biosystems, Foster City, CA). The analyte was characterized by atmospheric pressure chemical ionization multiple reaction monitoring. Quantitative drug concentrations were determined by standard calibration curve analysis, using linear fitting with 1/x² weighted plot of the analyte/IS peak area ratio vs analyte concentration.

**Subjects and clinical study procedures.** The clinical study protocol, informed consent document, and appropriate study-related documents were reviewed and approved by London-Riverside Research Ethics Committee (independent ethics committee) and the United Kingdom Medicines and Healthcare products Regulatory Agency. The study was conducted in compliance with the Declaration of Helsinki, International Council for Harmonisation Guideline for Good Clinical Practice, and applicable regulatory requirements. All study subjects provided written informed consent prior to the start of any study-specific procedures. Forty-eight healthy volunteers were enrolled into a double-blinded, randomized, placebo-controlled study (NCT03554993). The study was conducted at a single clinical site in the United Kingdom between May 2018 and July 2019. Single, ascending, oral doses of CC-99677 or placebo were administered to each subject in the fasted state once on the morning of Day 1 of the study. Subjects were randomized 6 active to 2 placebo in cohorts of 8 subjects receiving dose levels ranging from 3 to 400 mg. Subjects were domiciled at the clinical site during the dosing period and for several days afterward for daily clinical safety, PK, and pharmacodynamic assessments. Safety assessments, including physical exams, clinical laboratory tests, and collection of AEs, were performed throughout the course of the study.

**Clinical pharmacokinetic and pharmacodynamic sampling and analysis.**

*Pharmacokinetic sampling from healthy volunteers.* Blood samples were collected for determination of CC-99677 plasma concentrations prior to dose (0 hour) and at protocol-specified time-points up to 72 hours post-dose.

*Target engagement assay.* Target engagement of MK2 by CC-99677 was measured in PBMCs isolated from whole blood. Target engagement was assessed with the SMaSh assay. Blood samples for PBMC isolation were collected predose (0 hour) and at 4, 8, 24, 48, and 72 hours post-dose. Additional samples were collected on days 5, 7, and 14 for doses 30 mg and higher. Samples were evaluated for percent of MK2 bound to CC-99677 vs percent of free MK2 in PBMC lysates at each dose level. Target engagement was measured by calculating the change in percent bound MK2 from baseline.
Pharmacodynamic assessment from healthy volunteers. Effects of CC-99677 were assessed on cytokine production following ex vivo LPS stimulation using the TruCulture assay system (Myriad RBM, Austin, TX) to activate innate cells. Blood samples were collected predose (0 hour) and at 4, 8, 24, 48, and 72 hours post-dose. Additional samples were collected on days 4, 6, and 14 for doses 30 mg and higher. Briefly, blood (1 mL) was drawn into the LPS containing TruCulture tubes and the tubes were incubated at 37°C for 24 hours. In each dose-level cohort, inhibition of TNFα production in the ex vivo LPS stimulated blood was measured as percentage change in TNFα levels from baseline.

Analytical methods for CC-99677 human plasma. Concentrations of CC-99677 in human plasma from oral administration were analyzed using a validated LC-MS/MS method. Plasma samples were spiked with stable labeled CC-99677 as an IS. CC-99677 and its IS were extracted by liquid-liquid extraction using 750 μL ethyl acetate. After drying down, samples were reconstituted with 150 μL of ACN:H2O (22.5:77.5, v/v). The samples were injected for LC-MS/MS analysis using a Phenomenex Kinetex XB-C18 analytical column (50×2.1 mm, 1.7 μm) and mobile phases of A (10 mM ammonium format with 0.2% formic acid in H2O) and B (0.2% formic acid in acetonitrile). Positive ions were measured in the multiple reaction monitoring mode (m/z = 476.4 → 365.1 for CC-99677 and m/z = 476.4 → 367.1 for IS) using an API 5000 tandem mass spectrometer equipped with a TurboIonspray source. The lower limit of quantification for CC-99677 in human plasma was 0.5 ng/mL, with calibrated range demonstrated to 500 ng/mL. The inter-assay coefficient of variance (% CV) values, based upon the accepted calibration standards across the range, were ≤5.7% for CC-99677. Inter-assay precision values of CC-99677, based upon the % CV of the quality control (QC) samples, were ≤13.8%. Inter-assay accuracy values of CC-99677, based upon percent relative error (% RE) of the QC samples, ranged from −4.5% to −1.3% for all QC levels. All plasma samples were analyzed by Covance (Harrogate, UK).

RESULTS

Electrophile optimization and discovery of CC-99677. To identify a potent MK2 inhibitor, we focused first on solving the BE problem previously encountered with orthosteric inhibitors of the kinase. Analysis of the MK2 binding site reveals a shallow and narrow ATP binding pocket, hence the high aromatic character of published inhibitors. To increase affinity beyond leveraging the hydrophobic collapse and limited hydrogen bonding opportunities of purely reversible ligands, we pursued potent reversible ATP-competitive binding coupled with an electrophilic moiety capable of covalently bonding with the single ATP-site proximal C140. Unfortunately, C140 occlusion by residues asparagine 142 and lysine 89 and its role as a key hydrogen bond partner for most published inhibitors precluded using sterically demanding aliphatic acrylamides as electrophiles. Instead, we chose halogenated pyrimidines, which have minimal steric demands and tunable reactivity, making them ideal therapeutically compatible electrophiles for MK2 inhibitors. We were encouraged by the employment of p-fluoronitro arenes as targeted covalent inhibitors and envisioned extension of the SNAr mechanism to halo pyrimidines would afford a drug-like alternative to nitrohalo arenes.

For several reasons, we focused our attention on the tetracyclic scaffold in Table I as the ATP site binder for attaching a halopyrimidine electrophile. A low-resolution (3.7 A) co-crystal structure of MK2 with the benzothiophene PF-3644022, where R = 4-methyl-3-pyridyl for the scaffold of Table I, places the flat, mostly planar tetracyclic ring structure in the ATP binding cleft. The diazepinone ring is deeply buried in the back of the cleft and the pyridine ring lies near the hinge region. The 4-methyl-3-pyridyl ring is not critical for reversible potency, thus providing a vector to link an electrophile directed toward C140 for reaction. PF-3644022 was extensively characterized; however, with lower potency in cells, low safety margins in animals, and high dose projections, further development was halted. Attempts to achieve tighter intrinsic binding to allow for lower doses failed and the authors concluded that further cell potency optimization of this reversibly binding inhibitor was unlikely.

Initial docking showed that connecting a halo pyrimidine electrophile via a linking nitrogen (compound 1) or oxygen (compound 3) atom to the tetracyclic core (Table I) placed the reactive center of the chloropyrimidine near C140 (Fig 1, A). Both make hydrogen bonds to the hinge via interaction with the backbone NH of Leu141, Lys93 ε amine, and Asp207 γ carboxylate (of the DFG motif). These examples are both 2,4-substituted pyrimidines and, like the other benzothiophenes in Table I, share a methyltetrahydrodiazepinone (MTHD) ring. In parallel, we also explored 4,6-substituted electrophiles and variations of the benzothiophene scaffold. An early compound, a 4,6-substituted fluoropyrimidine with a primary amine on the MTHD ring (inset, Fig 1, B), showed 71% mass modification of recombinant MK2 detected by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS). Though a potent inhibitor of MK2
Fig 1. Tetracyclic inhibitors bound to MK2 (shown at scale). A, Models of the N-linked compound 1 (green) and O-linked compound 3 (yellow) docked to a 2.1 Å resolution structure of MK2 from 1B. Reversible ligands were minimized at a flexible active site of MK2 using Schrodinger software. B, Crystal structure of a covalent complex between the amino-MTHD (inset) and MK2. The overall co-crystal structure is in accordance with the published MAPKAPK2 kinase domain structure in complex with inhibitor (pdb entry 3M2W) with an r.m.s.d. of 0.22 Å considering 290 Cα atoms.
enzymatic activity ($IC_{50} = 12.1 \text{ nM}$), it did not inhibit the phosphorylation of HSP27 ($EC_{50} > 3 \text{ \mu M}$), an immediate downstream MK2 substrate, in cells (data generated in parallel with compounds listed in Table I). We were able to obtain a 2.1 A resolution X-ray structure of the covalent adduct of this amino-MTHD with MK2 (Fig 1, B). The amino-MTHD moiety is positioned beneath the glycine rich loop (not visible) thereby establishing a network of hydrogen bonds including Lys93 out of the N-terminal subdomain, Asp207 of the DFG motif and Glu190 out of the C-terminal subdomain. The 4-fluoropyrimidine moiety has reacted and covalently bonded with the side chain of C140 of the hinge. The 2Fo-Fc map (Supplemental Fig. 4) of the amino-MTHD in the ATP binding site shows electron density for this thioether linkage, providing confidence in this molecular architecture and in designing analogs that could address BE. Though the amino-MTHD scaffold provided requisite properties for X-ray crystallographic studies, it did not demonstrate cellular activity. We therefore focused on the parent MTHD scaffold in Table I for progression.

Key analogs informed our path to cell potency. Amine linked 2-chloropyrimidine (compound 1) potently ($IC_{50} = 35 \text{ nM}$) inhibits recombinant kinase activity, demonstrating high affinity for MK2. However, due to low intrinsic reactivity, no covalent adduct was observed via MALDI MS; this was reflected in a poor cellular EC$_{50}$. Introducing electron withdrawing groups, like a carboxamide, para to the electrophilic carbon (compound 2) had a dramatic effect on cellular potency. This effect can be traced to robust covalent modification of recombinant MK2 (85%) and increased biochemical potency ($IC_{50} = 4.7 \text{ nM}$). However, the N-linked series typically had poor physicochemical properties like low passive permeability; high efflux ratio in a Caco-2 model of intestinal epithelium, attributed to the free hydroxyl on the warhead linking aniline; and poor solubility. In line with this, amide compound 2 contains 2 additional H-bond donors and exhibits poor permeability and a high efflux ratio. By editing the linking atom to an oxygen, we achieved better passive permeability, A → B of 5 (compound 1) and 0.11 (compound 2) increases to 13 (compound 3). Aqueous solubility is also increased while maintaining good target modification and potency. This came at the cost of decreased metabolic (microsomal) and physical stability. Chemical stability studies indicated that hydrolysis at C-4 of the pyrimidine leading to loss of the electrophilic ring was occurring under acidic conditions. We reasoned that a substituent at the five-position of the pyrimidine would increase chemical and metabolic stability as well as provide an additional handle for property modulation. Addition of substituents containing a tertiary amine retained mass modification and potency while leading to significant improvements in the solubility, as exemplified by compound 4. Although this improved stability for some analogs, it was not universal and had a deleterious effect on passive permeability. Introduction of the benzylic alcohol (compound 5) improved metabolic stability, mouse S9 microsomal stability increased from 63% for compound 4 to 89% for compound 5 but decreased permeability and cell potency, most likely because of the polarity of the hydroxyl group. By capping the benzylic alcohol with an ether (CC-99677) we achieved a good balance between potency, reactivity, stability, and passive permeability.

CC-99677 represents a series of compounds that allowed modulation of the reactivity and provided stability of the chloropyrimidine electrophile by blocking C-4 from hydrolysis while still moderately activating the C-2 position for S$_9$Ar via inductive effects. This also maintained the passive permeability of the parent compound 3, a key to retaining oral bioavailability. Compounds within the series achieve “good” biochemical efficiencies, defined as $>0.4$, a guideline consistent with a majority of 50 successful therapeutic agents. This series also demonstrated adequate exposure to assess in vivo pharmacology and safety by oral administration. Because CC-99677 itself possessed the most attractive overall profile, we characterized it further.

**CC-99677 irreversibly binds MK2.** A mass shift in the matrix-assisted laser desorption/ionization (MALDI) spectrum of the full-length protein treated with excess inhibitor (10x for 1 hour) shows that CC-99677 (Fig 2, A and B) modifies MK2 covalently (≈74%). Analysis of the trypsin-digested complex identified a peptide of mass 2388 amu, the mass of the C140-containing peptide $^{133}$CLLLLVECLDGGLFSR$^{140}$ modified by the mass of inhibitor in which the chlorine atom has been displaced (Fig 2, C). This peptide was then selected in the mass spectrometer for tandem mass spectrometry (MS/MS) sequencing analysis. A total of 2 b and 9 y observed ions serve to positively identify C140 as the modified amino acid (Supplemental Table 2).

**Biochemical potency, mechanism, and cellular potency.** CC-99677 potently inhibits MK2 enzymatic activity with an $IC_{50} = 156.3 \pm 5.5 \text{ nM}$ (Table II, Fig 3, A). An extended kinetic analysis provides orthogonal evidence that the compound irreversibly inhibits the enzyme via a 2-step mechanism of action in which an initial reversible binding is followed by a covalent bond formation (Eq. 4).

$$E + I \xrightarrow{K_I} [E \cdot I] \xrightarrow{k_{	ext{on}}/[E-I]} [E-I]$$ (4)

Fitting the data to this kinetic model yields the potency of the initial binding, $K_I = 171.7 \pm 71.6 \text{ nM},$
Fig 2. CC-99677 covalently binds MK2. A, The mass spectrum of human recombinant MK2 protein. B, The mass spectrum of human recombinant MK2 incubated for 1 hour at room temperature with CC-99677 (MW = 469.84) showing a mass shift of 320 Da (74%), corresponding to significant covalent bonding of the inhibitor to MK2. Representative figures are shown. The m/z values of 49,189 and 49,509, are the averages of n = 3 determinations for MK2 and MK2 + inhibitor, respectively. C, CC-99677 modifies cysteine 140 of MK2. MK2 treated with a molar excess of CC-99677 for 1 hour at room temperature was reduced, alkylated, and then digested with trypsin. The resulting peptides were analyzed by MALDI. The MS/MS spectrum of one 2388 Da peptide (inset) matches the predicted theoretical mass expected for the target peptide covalently modified upon reaction with MK2. Labeled are the mass to charge ratios of 2 b and 9 y ions matching predicted peptide fragments. (Color version of the figure is available online.)
Fig 3. CC-99677 irreversibly inhibits MK2 kinase activity. A continuous fluorescence read assay was used to monitor the phosphorylation of a Sox peptide substrate by MK2. A. Dose response curve of initial enzyme activity normalized to enzyme only and fit to Equation 1 to obtain IC$_{50}$ = 155.3 nM and Hill Slope = 0.86. B. The non-linear progress curve for 8 inhibitor concentrations was fit to an ascending single exponential equation to yield an observed rate, $k_{obs}$. The dose dependence of $k_{obs}$ was then fit to Equation 2 to yield the reversible initial inhibitor binding interaction $K_i$ = 230 ± 34 nM and the inactivation rate constant $k_{\text{inact}}$ = (10 ± 0.4) x 10$^{-4}$ s$^{-1}$. The overall reaction rate = $k_{\text{inact}}/K_i$ = 4.3 x 10$^3$ M$^{-1}$s$^{-1}$. A and B, each shows 1 representative dose response curve. The experiments were performed in triplicate and the average of the 3 individually fitted values reported in Table II.
and the rate of the irreversible bond formation or inactivation step, $k_{\text{inact}} = 8.2 \times 10^{-4} \text{s}^{-1}$ ($n = 3$; Table II, Fig 3, B). The overall biochemical potency is $k_{\text{inact}}/K_I = (4.94 \pm 0.63) \times 10^3 \text{M}^{-1}\text{s}^{-1}$. This second step makes the mechanism of action time dependent. Hence, IC$_{50}$, although sometimes convenient, is a limited kinetic characterization.

CC-99677 potently inhibited HSP27 phosphorylation (pHSP27) with an EC$_{50}$ (dose at which 50% of the pHSP27 formation is inhibited) of $89 \pm 2.6 \text{nM}$ and an OC$_{50}$ (dose at which 50% of the cellular MK2 is occupied) of $164 \pm 18 \text{nM}$ (Table II, Fig 4) as determined by Streptavidin Mass Shift (SMaSh), a Western-based mass shift assay, described further in Material and Methods (Supplemental Fig. 5). It is notable that 100% free protein was not observed in the absence of inhibitor reflecting an incomplete ability of the probe to saturate all the MK2 in the cell. Nonetheless, the inflection for both the functional inhibition of pHSP27 and drug occupancy were close in value.

Using the ratio of the biochemical IC$_{50}$ and cellular OC$_{50}$, BE$^{OC} = 0.95$. Alternatively, using cell potency, EC$_{50}$, as a measure of function instead of occupancy, BE$^{EC} = 1.76$. By either assessment CC-99677 demonstrates BE $> 0.4$.

Many factors can determine BE; permeability is critical. Consistent with the high BE, CC-99677 exhibits good permeability in CACO-2 cells, A $\rightarrow$ B: $21 \times 10^{-6} \text{cm/s}$, B $\rightarrow$ A: $48 \times 10^{-6} \text{cm/s}$, with an efflux ratio 2.3. By contrast, the benzylic alcohol (compound 5) appears even more potent biochemically than CC-99677, IC$_{50} = 115 \text{nM}$, but is less potent in cells, perhaps because of a low permeability and a high efflux ratio (Table I).

### Table II. Measures of biochemical and cellular MK2 inhibition by CC-99677

<table>
<thead>
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<th>Measure</th>
<th>Value</th>
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<tr>
<td>IC$_{50}$</td>
<td>156.3 $\pm$ 5.5 nM</td>
</tr>
<tr>
<td>$K_I$</td>
<td>171.7 $\pm$ 71.6 nM</td>
</tr>
<tr>
<td>$k_{\text{inact}}$</td>
<td>$(8.2 \pm 2.7) \times 10^{-4} \text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{inact}}/K_I$</td>
<td>$(4.94 \pm 0.63) \times 10^3 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>OC$_{50}$</td>
<td>164 $\pm$ 18 nM</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>89 $\pm$ 2.6 nM</td>
</tr>
</tbody>
</table>

Abbreviations: EC$_{50}$, half-maximal effective concentration; IC$_{50}$, half-maximal inhibitory concentration; OC$_{50}$, dose at which 50% of MK2 is occupied.

All values are the mean $\pm$ standard deviation derived from the average of 3 independent experiments. The data from each individual experiment were separately fit to the appropriate equation and the fitted values from each of the 3 experiments then averaged.
**Table III. Human recombinant kinases inhibited >80% at 1 μM CC-99677 and corresponding IC<sub>50</sub> values**

<table>
<thead>
<tr>
<th>Kinase</th>
<th>% Inhibition</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Selectivity&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DYRK1/DYRK1A</td>
<td>98</td>
<td>5</td>
<td>0.03</td>
</tr>
<tr>
<td>DYRK3</td>
<td>98</td>
<td>6</td>
<td>0.04</td>
</tr>
<tr>
<td>ERK5/MAPK7</td>
<td>97</td>
<td>32</td>
<td>0.21</td>
</tr>
<tr>
<td>LRRK2</td>
<td>91</td>
<td>42</td>
<td>0.27</td>
</tr>
<tr>
<td>p70S6Kb/RPS6KB2</td>
<td>89</td>
<td>71</td>
<td>0.46</td>
</tr>
<tr>
<td>MYO3b</td>
<td>90</td>
<td>79</td>
<td>0.51</td>
</tr>
<tr>
<td>KSR2</td>
<td>87</td>
<td>87</td>
<td>0.56</td>
</tr>
<tr>
<td>MAPKAPK5/PRAK</td>
<td>85</td>
<td>101</td>
<td>0.65</td>
</tr>
<tr>
<td>CAMKK2</td>
<td>82</td>
<td>140</td>
<td>0.90</td>
</tr>
<tr>
<td>MAPKAPK2 (MK2)</td>
<td>94</td>
<td>156</td>
<td>1.00</td>
</tr>
<tr>
<td>DYRK1B</td>
<td>83</td>
<td>170</td>
<td>1.09</td>
</tr>
<tr>
<td>KSR1</td>
<td>85</td>
<td>190</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Abbreviations: IC<sub>50</sub>, half-maximal inhibitory concentration.<br>Dose titrations to determine IC<sub>50</sub> were performed using a Miniaturized HotSpot<sup>™</sup> assay and an ATP concentration equivalent to the Km for ATP of each kinase. The average of 2 determinations was rounded to the nearest integer.<br><sup>†</sup>Selectivity = IC<sub>50</sub> of kinase in the list/IC<sub>50</sub> of MK2.

In vitro safety pharmacology and kinase selectivity panel. No significant interactions were deemed likely, at pharmacologically relevant concentrations, in binding competition assays using an in vitro safety pharmacology panel of 80 receptors using radioisotopically labeled ligands.

CC-99677 is biochemically selective when tested against a panel of 364 kinases at their individual Km ATP concentrations (described in Material and Methods, full data set in Supplemental Table 3). However, it did inhibit 11 off-target kinases >80% and 22 off-target kinases >50% at 1 μM. The corresponding IC<sub>50</sub> values for the former are shown in Table III. Of importance, no significant inhibition was observed for the 4 isoforms of p38 tested (p38α, β, δ, and γ; Supplemental Table 3). There was no selectivity against 4 of the 11 kinases inhibited and only 2 to 5-fold selectivity against the other 7. Five kinases share a structurally homologous cysteine to C140: p70S6Kb, MAPK-activated protein kinase-3 (MK3), fibroblast growth factor receptor 4 (FGFR4), serine/threonine kinase 40 (STK40), and tyrosine threonine kinase (TTK). p70S6Kb, MK3/MAPKAPK3, and FGFR4 are inhibited 89% (Table III), 20%, and 73% (Supplemental Table 3), respectively. We observed previously that biochemical potency for an off-target does not always translate into cells. Therefore, we assessed cellular activity of 6 kinases from Table III that were amenable to quantify in a cellular-based readout: dual specificity tyrosine phosphorylation regulated kinase (DYRK)1A, DYRK1B, extracellular signal-regulated kinase 5 (ERK5), leucine-rich repeat kinase 2 (LRRK2), p70S6Kb, and calcium/calmodulin-dependent protein kinase 2 (CAMKK2). No functional inhibition of these targets by the MK2i was observed in intact cells (Supplemental Fig. 6). We do not know if CC-99677 is active intracellularly against other kinases on the list.

Effect of CC-99677 vs p38 inhibitors on LPS-activated human PBMCs. The rationale for targeting MK2 is to suppress inflammatory cytokines controlled by the p38 pathway but spare p38 itself to avoid negative outcomes, like tachyphylaxis. We reasoned that if the cellular consequences of inhibiting these two pathway member enzymes differ then, perhaps, MK2 inhibition would not simply reproduce the same responses as p38 inhibition. Therefore, we profiled the production of 5 key cytokines upon LPS stimulation of human peripheral blood mononuclear cells (PBMCs) in the presence of: the MK2i (CC-99677), two p38 inhibitors (SCIO-469/talmapimod or BMS-582949), or a JAK1/JAK3 inhibitor (tofacitinib).

We observed a different pattern of IL-1β and monocyte chemoattractant protein 1 (MCP-1) inhibition for CC-99677 and the p38 inhibitors (Fig 5). The p38 inhibitors potently inhibited IL-1β (IC<sub>50</sub> ≤ 5 nM), whereas CC-99677 had minimal effect (IC<sub>50</sub> > 3000 nM) (Fig 5, A). By contrast, MCP-1 was inhibited by CC-99677 (IC<sub>50</sub> = 265 ± 23 nM) but significantly upregulated by p38 inhibitors (Fig 5, B). CC-99677 potently inhibited TNFα and GM-CSF (IC<sub>50</sub> = 67 ± 15, IC<sub>50</sub> = 258 ± 29 nM, respectively; Fig 5, C and D) and moderately inhibited IL-6 (IC<sub>50</sub> = 865 ± 26 nM; Fig 5, E). The observed effects of CC-99677 on cytokine secretion were similar to those seen with the known MK2 inhibitor PF-364022 (Supplemental Fig. 7). As expected, tofacitinib had no effect on production of most cytokines in LPS-activated monocytes. These results show MK2 inhibition differs from p38 inhibition.

The slow resynthesis rate of MK2 is compatible with a TCI. Pursuit of a TCI to address potency is optimal for a protein target with a slow resynthesis rate, as it minimizes competition with de novo protein synthesis. This is expected to reduce the need for sustained pharmacokinetics to maintain inhibition. The MK2 t½ was determined in adherent THP-1 cells, activated with phorbol myristate acetate (PMA), by stable isotope labeling with amino acids in cell culture (SILAC) in the absence and presence of CC-99677 at 900 nM, ~ 10 x EC<sub>50</sub>. The long t½ of MK2, 44 ± 7 hours, was unaffected by the presence of the TCI, t½ (+ inhibitor) = 38 ± 4 hours. Both are averages of 3 independent determinations ± SEM (see Supplemental Fig. 8 for example).

A washout experiment was also used to estimate resynthesis half-life as an orthogonal approach. Adherent THP-1 cells were treated with CC-99677 for 1 hour then repeatedly washed to remove any excess...
Fig 5. CC-99677 exhibits a distinct anti-inflammatory profile in LPS-stimulated PBMCs. The effects of MK2 inhibition in healthy human donor PBMCs was assessed by monitoring 5 proinflammatory cytokines: IL-1β (A); MCP-1 (B); TNFα (C); GM-CSF (D); and IL-6 (E). The cells were exposed to varying concentrations of the inhibitor for 1 hour at 37˚C and then stimulated by LPS for 48 hours at the same temperature. Three other anti-inflammatory inhibitors were included for comparison, 2 p38 inhibitors, SCIO-469 and BMS-582949, and the JAK3 inhibitor, tofacitinib. EC_{50} shown as mean (standard deviation). Mean values for n = 4 donors are plotted with error bars representing SEM. (Color version of the figure is available online.)
compound. Resynthesized MK2 was then followed by the SMaSh assay, which uses a covalent inhibitor analog as probe (Supplemental Fig. 3) to quantify inhibitor occupancy (see Material and Methods for protocol).38 Here it was used to quantify newly synthesized protein. Just after the washout, little free MK2 remained but recovered to 50% in ~43 hours (Supplemental Fig. 9), in good agreement with the half-life obtained from SILAC. We also examined non-adherent THP-1 and other cell types, PBMCs, monocytes, and naive CD4+ T cells, and found that MK2 displayed a similarly long half-life in all cases. A similar determination of PBMCs in rat, in anticipation of running a rat disease model, found MK2 t_{1/2} ~27 hours. This experiment reveals a slow MK2 resynthesis rate, a critical determinant for a prolonged duration of action resulting from target silencing via an irreversible inhibition.

**In vivo efficacy in a rat model of AS.** We used male HLA-B27/Huβ2m transgenic rats as a model of AS. These rats express multiple copies of the MHC I allele, HLA B27, an allele that is associated with AS in humans.40-43 Biologic agents demonstrating disease-modifying activity in human clinical trials (anti-TNF44 and anti-IL1745) have also shown activity in this rat model of spondyloarthritis.42,43 The JAK inhibitor, tofacitinib, which showed clinical activity in human AS,46 served as a comparator. The resulting arthritis of the model manifests as paw swelling, which was scored according to van Tok et al (2017).

CC-99677 elicited dose-dependent inhibition of paw swelling (Fig 6, A). A 20 mg/kg dose resulted in a 41% reduction in paw swelling, derived from the AUC of clinical paw swelling scores as described in the Materials and Methods (Fig 6, B). At 24 hours target occupancy was 23% and 53% in spleen and PBMCs, respectively. At the 100-mg/kg dose, paw swelling was further reduced to 65% and target occupancies were higher, 30% and 72% for spleen and PBMCs, respectively. SMaSh showed high target occupancy in extracts from PBMCs and spleen (Supplemental Fig. 10). Prior PK studies in rats demonstrated oral bioavailability. For the most relevant data for this preclinical model, blood was collected after the last dose of the study to confirm and assess exposure. CC-99677 showed less than dose-proportional exposure following administration of 20 and 100 mg/kg daily doses orally following 35 days of dosing (Supplemental Table 4). Despite the relatively short half-life (2.8 and 3.6 hours, respectively) in vivo, the exposures were efficacious.

**Human pharmacokinetics and pharmacodynamics.** A phase 1, randomized, double-blind, placebo-controlled, single-ascending-dose study evaluated CC-99677 in 48 healthy volunteers (46% male, 54% female, 85% white; mean age 31 years [range: 20–53]). No safety stopping criteria were met, and all adverse events (AEs) were mild except 1 moderate gastroenteritis event, with no apparent dose dependency. There were no dose-related trends in clinical laboratory results, vital signs, or electrocardiogram results. Of the 36 subjects who received CC-99677, 10 (27.8%) experienced at least 1 AE vs 5 (41.7%) of the 12 placebo subjects. In 60% of the subjects receiving CC-99677 who experienced an AE, no relationship between the AE and study drug was suspected by the blinded investigator. This includes no relationship between the study drug and the moderate episode of gastroenteritis. In the CC-99677 group (n = 36), mild somnolence and mild nausea (n = 1 each at the 100 mg dose level), and 2 mild, transient, rashes without other organ involvement at the 400 mg dose level were suspected of being related to the study drug by the blinded investigator.

The PK characteristics of CC-99677 showed a linear increase in exposures, area under the curve (AUC), from 3 to 400 mg. CC-99677 was absorbed with a time to median maximum plasma concentration (T_{max}) of 1 to 3 hours as the dose level progressed from 3 to 400 mg (Fig 7, A). The geometric mean t_{1/2} ranged from 1.7 to 1.9 hours from 3 to 10 mg, from 2.7 to 3.1 hours from 30 to 100 mg, and from 4.9 to 6.9 hours from 200 to 400 mg. By 24 hours, there was minimal detectable plasma CC-99677 at all doses. We also assessed MK2 kinase occupancy, the most proximal pharmacodynamic marker. Maximal target engagement was observed at 8 hours (Fig 7, B; SMaSh occupancy data for the 100 mg dose is shown in Supplemental Fig. 11). Median MK2 occupancy increased from 8.6% to 82.7% as the dose was increased from 10 to 400 mg (Fig 7, B). There was no significant occupancy observed at 3 mg. MK2 occupancy returned to pre–dose baseline levels by Day 14.

Given that single doses of CC-99677 could achieve target engagement levels associated with improved paw swelling in the AS animal model and that target engagement was sustained even after plasma concentrations of CC-99677 were no longer detectable, we evaluated the effect of CC-99677 on proinflammatory cytokine levels. TNFα is a key cytokine controlled by MK2 that has been successfully targeted therapeutically for many rheumatologic diseases, including AS. TNFα protein secretion from ex vivo LPS-stimulated whole blood from healthy volunteers was inhibited within 4 hours after a single dose of CC-99677 from 10 to 400 mg (Fig 7, C). This inhibition
Fig 6. CC-99677 inhibits paw swelling in a rat model of AS. Arthritis was induced in male HLA-B27/Huβ2m rats by injection of CFA. Vehicle or compound was administered 5 days post CFA and clinical arthritis scores recorded thrice weekly for 35 days. A, Clinical arthritis scores based on paw swelling for vehicle, 2 doses of CC-99677 and tofacitinib as a positive control. Error bars represent SEM. B, AUC of the paw swelling time curves for vehicle and each of the 3 treatments. Percent inhibition above each bar is the difference between vehicle and treatment normalized to vehicle. Differences between treatment groups were determined by calculating AUC of clinical scores and analyzing data by ANOVA with Kruskal Wallis post hoc. Error bars represent SEM. 

\[ N = 9 \text{ for each treatment group; there was 1 death for the tofacitinib group, so the study ended with 8 animals.} \]

Route of administration and schedule are: vehicle PO, QD; 20 & 100 mg/kg CC-99677 PO, QD; tofacitinib PO, QD. PO=orally; QD=once daily. Asterisks over the arrow denote the overall significance of ANOVA; asterisks over individual groups denote the significance of each treated group vs vehicle. \[ *P < 0.05; \ ***P < 0.001. \]

(Color version of the figure is available online.)
Fig 7. CC-99677 human pharmacokinetics, MK2 occupancy, and TNFα inhibition. A, Pharmacokinetics. Each cohort of 6 human healthy volunteers were administered 1 oral dose of CC-99677. Blood concentration of the drug was measured prior to dosing and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours (48 & 72 not shown). Error bars represent the SEM; no error is represented for the lower doses as they are too small to visualize. B, MK2 occupancy. Using the SMaSh assay, MK2 occupancy was determined in isolated PBMCs from blood drawn prior to dosing (t = 0) and at the indicated times shown. The % change is background corrected for a population of MK2 that is not shifted by the probe. C, TNFα Inhibition. The % change in TNFα production determined ex vivo in whole blood using LPS stimulation in a Tru-Culture tube between predose (t = 0) and 4, 8, and 24 hours for each dose. The median of the values for the cohort are plotted in B and C and the error bars represent the interquartile range. For placebo n = 12. For all other cohorts n = 6.
was sustained for up to 48 hours. Ex vivo-stimulated whole blood from subjects dosed below 10 mg of CC-99677 (including placebo) still retained the diurnal spike in TNFα secretion.51

**DISCUSSION**

The p38 pathway is central to the inflammatory response; however, attempts to inhibit it directly for therapeutic benefit have repeatedly failed, largely for safety reasons. The few compounds safe enough for clinical testing exhibited tachyphylaxis, which has been attributed to futile interference with p38 feedback mechanisms that balance inflammatory and anti-inflammatory signaling. Tachyphylaxis was not observed in preclinical animal models, so it was unanticipated in the clinic.52,53 Inhibition of an immediate downstream p38 substrate, MK2, has long been postulated as a means of circumventing feedback futility. We demonstrated that p38 and MK2 inhibition have different cytokine profiles and that CC-99677 inhibits monocyte activation. In contrast to p38 inhibitors, MK2 inhibitors MCP-1 production. Breakdown of a negative feedback mechanism mediated by MAPK phosphatase-1 (MKP-1), which inactivates p38α and JNK immune responses, may explain the inability of p38 inhibitors to affect MCP-1 production. Pharmacological and genetic studies demonstrated that intact MKP-1 activity is essential for suppression of MCP-1 production in macrophages.54-57 Importantly, MCP-1 stimulation can induce MK2 activation in myeloid cells,58 therefore, the apparent increase in MCP-1 production after treatment with p38 inhibitors may further diminish the downstream anti-inflammatory effect by a cell autonomous mechanism.

Overall, CC-99677 displays broader anti-inflammatory inhibitory activity on monocyte activation than p38 inhibitors and tofacitinib. Although the distinct cytokine profile of MK2 inhibition does not guarantee sustained suppression of inflammation, it does demonstrate that reversible p38 and irreversible MK2 interventions are different.

Identifying a clinical compound to develop has been challenging. Most efforts have focused on orthosteric binders that pit the enzyme’s low micromolar ATP affinity against millimolar cellular ATP. Many of these exhibited poor BE and were not developed further. We addressed these challenges by designing a targeted covalent orthosteric inhibitor.

The chloropyrimidine of CC-99677 irreversibly bonds to and inhibits MK2 via an SNAr molecular mechanism, translating biochemical potency into the cell. The planar tetracyclic scaffold initially docks the molecule into the ATP binding site, binding with a potency (Kᵢ) of 170 nM. Once docked, the electrophile is proximal to C140 on the kinase domain hinge and enables the displacement reaction, in which a chlorine atom is replaced by a sulfur atom of C140, creating a thioether bond between compound and protein. Covalency provides a means of successfully competing with high cellular ATP concentrations. The resulting BE is near (0.95) or exceeds (1.76) the ideal value of 1, depending on how it is calculated; both exceed the 0.4 guideline consistent with successful drugs.59 The cellular potency, EC₅₀, is equal to or greater than the biochemical IC₅₀ potency. We have observed this previously with other kinase TCIs and it is likely a result of the covalent and time dependent nature of the mechanism. To our knowledge, it is the first time this specific chloropyrimidine electrophile and SNAr mechanism have been harnessed in the clinic.

Besides addressing BE, the permanent nature of a covalent bond lends itself to multiple approaches of establishing and quantifying target occupancy. Here we use both mass modification by mass spectrometry (MS) and the SMaSh assay. The former, primarily used with recombinant isolated enzyme, provides a facile means of quickly assessing covalent effectiveness, thereby guiding design. The SMaSh assay is a user-friendly tool for quantitatively tracking target engagement in cells.38 Because the assay also works in tissues it provides a pharmacodynamic biomarker for preclinical and clinical studies and is a means of connecting target engagement to efficacy, thereby aiding clinical dose determination. Knowing how much target engagement translates into cytokine inhibition and ultimately disease efficacy is empowering for clinical studies. This is especially useful as PK is uncoupled from pharmacodynamics and efficacy. Although exposure is required, it does not have to be sustained as it does for reversible inhibitors to achieve adequate target suppression. CC-99677 permanently disables enzyme activity requiring de novo synthesis of MK2 to replenish that activity. The resynthesis half-life of human MK2 in THP-1 cells or PBMCs is on the order of ~40 hours while the CC-99677 half-life in humans is ~2 to 7 hours, depending on dose. MK2 is irreversibly inhibited upon MK2i dosing, resulting in TNFα suppression for up to 48 hours even though the unbound inhibitor is cleared from circulation relatively quickly.

Orthosteric MK2 inhibitors like CC-99677 directly inhibit the enzyme’s ability to phosphorylate all substrates, one way of inhibiting downstream signaling. PoA MK2 inhibitors bind to p38 and can display p38 substrate selectivity (e.g., inhibit activation of MK2 but spare the activation of p38 substrates like mitogen and stress-activated protein kinase 1 [MSK1] or activating...
transcription factor 2 (ATF2)). Such selective inhibition may avoid toxicities and/or tachyphylaxis seen with many direct p38 inhibitors. However, those that bind to the p38 catalytic pocket and compete with ATP can be problematic and translate into lower BEs. Two such published compounds have good selectivity, 34 and 160-fold selectivity for MK2 vs MSK1, but translate poorly into cell activity with apparent BEs of 0.1 and 0.04, respectively.59 Another PoA inhibitor, ATI-450, spares the p38 substrates p38-regulated/activated protein kinase (PRAK) and ATF2 while effectively inhibiting MK2 activation.19,20 Successful competition with cellular ATP was not specifically assessed, but inhibition of MK2 activation was demonstrated in cells with consequent inhibition of TNFα, IL-6, and IL-1β production in LPS-stimulated bone marrow–derived macrophages from wild type and/or a neonatal-onset multisystem inflammatory disease (NOMID) mouse model. Both CC-99677 and ATI-450 prevent downstream signaling of MK2 by different molecular mechanisms. It is not yet clear if their resulting inhibitory cytokine profiles are similar. Both show efficacy in preclinical animal models of inflammation, albeit different models, a rat model of AS for CC-99677 (and 3 others not included here) and reduced inflammation in NOMID mouse model and rat SWC arthritis model for ATI-450.19 Both have completed first-in-human (FIH) clinical trials, and ATI-450 has completed a phase 2 study in RA.

Ankylosing spondylitis represents a patient population that could benefit from a greater range of therapeutic options. Conventional disease-modifying antirheumatic drugs vary in effectiveness, for example, methotrexate was not deemed effective60 whereas sulfasalazine can be used for peripheral arthritis.61 A recent longitudinal study shows that TNF blockers provide benefit by decreasing disease activity and are associated with a reduction in radiographic progression.60 An IL-17 inhibitor has recently been approved as a promising therapeutic approach. CC-99677 inhibition of MK2 offers the therapeutic benefit of suppressing TNFα, and the additional cytokine inhibitory activities could result in a small-molecule therapy differentiated from current AS treatment options.

Results of the FIH study for CC-99677 demonstrate that this oral covalent MK2 inhibitor can be dosed safely in humans, allowing further clinical development. Preclinical data show that it can reach MK2 occupancy levels associated with improvement in paw swelling in an animal model of AS. Clinical data from single-dose administration demonstrate that comparable occupancy levels can be achieved in healthy human volunteers. At those levels there is a concentration and occupancy-dependent, sustained decrease in TNFα in ex vivo stimulated human whole blood. Small cohort size likely is contributing to the large variability seen in TNFα suppression. Collectively, these data support the need for and utility in continued development of CC-99677 as an anti-inflammatory therapeutic.

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**SUPPLEMENTARY MATERIALS**

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2022.06.005.

**REFERENCES**


