p38α mitogen-activated protein kinase (MAPK) plays a role in several cellular processes and consequently has been a therapeutic target in inflammatory diseases, cancer, and cardiovascular disease. A number of known p38α MAPK inhibitors contain vicinal 4-fluorophenyl/4-pyridyl rings connected to either a 5- or 6-membered heterocycle. In this study, a small library of substituted thienophenyl-based compounds bearing the vicinal 4-fluorophenyl/4-pyridyl rings was designed using computational docking as a visualisation tool. Compounds were synthesised and evaluated in a fluorescence polarisation binding assay. The synthesised analogues had a higher binding affinity to the active phosphorylated form of p38α MAPK than the inactive nonphosphorylated form of the protein. 4-(2-(4-fluorophenyl)thiophen-3-yl)pyridine had a Ki value of 0.6 μM to active p38α MAPK highlighting that substitution of the core ring to a thiophene retains affinity to the enzyme and can be utilised in p38α MAPK inhibitors. This compound was further elaborated using a substituted phenyl ring in order to probe the second hydrophobic pocket. Many of these analogues exhibited low micromolar affinity to active p38α MAPK. The suppression of neonatal rat fibroblast collagen synthesis was also observed suggesting that further development of these compounds may lead to potential therapeutics having cardioprotective properties.

Introduction

p38α Mitogen-activated protein kinase (MAPK) is a serine threonine protein kinase that regulates multiple cellular processes. Although this ubiquitous kinase has primarily been targeted for its role in inflammatory diseases, p38α MAPK activity has also been linked to cardiovascular disease as it coordinates the myocardial stress response to various stimuli including inflammatory, ischemic, mechanical, oxidative, neurohumoral, and pharmacological stressors. Pharmacological inhibition of p38α MAPK has been shown to improve cardiac function and reduce cardiac remodelling post myocardial infarction or cardiac injury. The structure of p38α MAPK is typical of most protein kinases consisting of N- and C-terminal domains that are connected by a hinge region (His107–Asp112). The ATP binding site is formed by the interface between the two domains with the hinge region as its border. This binding site is made up of an adenine binding region, sugar pocket, phosphate binding region, and two hydrophobic pockets. One hydrophobic pocket contains a ‘gatekeeper’ residue. In p38α MAPK this residue is a threonine (Thr106), which is smaller than the corresponding methionine or glutamine seen in p38γ, p38δ, extra-cellular-signal-regulated kinases (ERKs), and C-Jun N-terminal kinases (JNKs). An example of a p38α MAPK inhibitor that utilises this pocket is SB203580 (Figure 1), which has an IC50 value of 74 nM. In this compound the 4-fluorophenyl substituent fits optimally in the hydrophobic pocket. Along with this key interaction, SB203580 contains a 4-pyridyl ring that occupies the adenine binding region and forms a key hydrogen bond with the Met109 amide in the hinge region. The vicinal 4-fluorophenyl and 4-pyridyl rings present in SB203580 are also found in many other p38α MAPK inhibitors and are often connected by a 5- or 6-membered heterocycle. Other interactions of SB203580 include a hydrogen bond between the imidazole core and the Lys53 side chain and π-stacking interactions between the 4-methylsulfinylphenyl moiety and the Tyr35 side chain in the phosphate binding region.

Although SB203580 inhibits the p38α and p38β MAPK isoforms and not p38γ or p38δ MAPK isoforms, it was later shown to interfere with other protein kinases. Other pyridyl imidazoles have displayed higher potency and selectivity including RWJ67657 (Figure 2), which has an IC50 of 30 nM. An
investigation into the specificity of RWJ67657 showed no inhibition of recombinant p38\(\gamma\) or p38\(\delta\) MAPK isoforms or a number of other protein kinases (ERK2, protein kinase A, p56\(\text{lck}\), and c-\(\text{src}\) tyrosine kinases). This compound contains a phenylpropyl group that is thought to bind in the second hydrophobic pocket. Goldstein et al. described structural features of the protein that may be targeted for additional selectivity. As well as the hydrophobic “gatekeeper” pocket, optimising inhibitor binding to the second hydrophobic pocket may contribute to selectivity.

Compounds were designed to target the ATP binding pocket. The diaryl-heterocycle p38\(\alpha\) MAPK inhibitor class was investigated in which compounds contained a thiophene core. Fujita et al. synthesised substituted thiophenes and bicyclic compounds possessing the key vicinal 4-fluorophenyl/4-pyridyl rings seen in many p38\(\alpha\) MAPK inhibitors. Their substituted thiophene ester shown in Figure 2, was found to suppress TNF-\(\alpha\) production with an IC\(_{50}\) value of 1.7 \(\mu\)M. By combining characteristics of this TNF-\(\alpha\) suppressor with features of a known p38\(\alpha\) MAPK inhibitor (RWJ67657, Figure 2), we aimed to synthesise thiophene-based p38\(\alpha\) MAPK inhibitors. We also sought to determine whether substitution of the core 5-membered heterocycle with a thiophene could retain p38\(\alpha\) MAPK activity. In addition, the second hydrophobic pocket was probed for further interactions using an extra aryl ring.

Computational modelling was used to dock in designed ligands and enable visualisation of their likely binding mode in the p38\(\alpha\) MAPK protein. A series of tetra-substituted thiophenes were synthesised and evaluated in a fluorescence polarisation binding assay. Their effect on cardiac fibroblast collagen synthesis was also determined.

### Results and Discussion

#### Molecular Modelling

Although there are more than 200 X-ray crystal structures of p38\(\alpha\) MAPK available, the structures exhibit a high degree of ligand-induced conformational changes. As such it was imperative to identify the binding conformation of the protein for the diaryl-heterocycle inhibitor class and to determine which crystal structure is most suitable for docking ligands into the protein. In a previous publication we identified a crystal structure model of p38\(\alpha\) MAPK using virtual screening and ensemble docking for the diaryl-heterocycle p38\(\alpha\) MAPK inhibitor class bearing 4-fluorophenyl and 4-pyridyl rings. The crystal structure model identified was an ensemble of the 1BL7 and 2EWA crystal structures, which was therefore used for our docking studies.

In this work 53 compounds were designed and docked into the 1BL7 and 2EWA structures, scoring them using our ensemble method. The designed ligands suggested for p38\(\alpha\) MAPK inhibition are described in the supporting information. These ligands contained the 4-fluorophenyl/4-pyridyl rings seen in many p38\(\alpha\) MAPK inhibitors. In addition a 2-butynyl alcohol substituent, similar to RWJ67657, was used to extend into the polar outer rim of the binding site. A fourth aromatic ring was substituted with hydrogen bond donor or acceptor groups to probe for extra interactions with the protein. Figure 2 illustrates the type of ligands designed and docked into the protein.

Docking was carried out using the Glide v5.6 (Schrödinger) extra precision (XP) method. For the ensemble analysis, the glide scores of the top ranked pose of each ligand in the 1BL7 and 2EWA structures were retained and averaged. The compounds were then re-ranked based on the calculated ensemble scores (see Supporting Information). A visual inspection of the docked compounds within the binding site enabled selection of compounds for synthesis. For example, the top ranked structure was analogue 37 (Figure 3), which contained a meta-(2-hydroxyethyl)carbamoyl moiety. Figure 3 shows the docked pose for analogue 37 in the 2EWA crystal structure in which an extra hydrogen bonding interaction is made with the Gly110 backbone amide. Also shown in Figure 3 is the docked pose of
analogue 46 bearing a para-toluenesulfonamide group. Overall, this compound ranked eighth and it was proposed that an extra hydrogen bond between the amide proton and the Asp112 side chain would be made while the extra toluene group fills the second hydrophobic pocket.

Synthesis

The target thiophenes were synthesised using a series of palladium-catalysed cross coupling reactions, outlined in Scheme 1. Starting with 2,3-dibromothiophene, a one-pot double Suzuki reaction was used to first couple 4-fluorophenylboronic acid, followed by pyridine-4-boronic acid. Coupling at the 3-position proceeded more slowly and required heating at a higher temperature, yielding compound 2 in 71% over the two steps. The thiophene was then halogenated for subsequent palladium-catalysed cross coupling reactions. An organomercury intermediate was used to increase the reactivity of the system to halogenation. Thiophene 2 was reacted with mercuric acetate in acetic acid at 70°C for 16 h. Once the mercuric acetate intermediate was isolated the compound was reacted with an aqueous solution of potassium triiodide affording 81% of the diiodinated product 3. Next, a Sonogashira or Negishi coupling reaction was conducted to introduce the alkyne functionality. The Sonogashira reaction was carried out by heating thiophene 3, tert-butyl(dimethyl)silane (TBS)-protected butynyl alcohol 5, bis(triphenylphosphine)palladium(II) dichloride, copper iodide, triphenylphosphine, and triethylamine in tetrahydrofuran (THF) to give intermediate 6 in 82% yield. Alternatively, the Negishi coupling reaction afforded the same product in 94% yield using thio-
ride, n

28 methanol at reflux gave the final products (n...tion of the TBS group using ammonium fluoride in

boronic acid under microwave irradiation. Deprotec-

phine)palladium(II) dichloride and an excess of the

the second hydrophobic pocket. The Suzuki coupling

mediate coupling reaction was preferred. With the key inter-

moisture. For ease of synthesis the Sonogashira

reaction was higher, the reaction was more sensitive

palladium(0). While the yield for the Negishi coupling

ortho hydrogen bond donor and acceptor groups in the

thesised from the Suzuki coupling of methylcarboxyphenylbor-

boxy- and amino-phenyl analogues were selected for

a result of our initial docking studies, a series of car-

logues were not synthesised.

In addition to the carboxy- and amino-phenyl analogues,

ortho positions of the aryl ring. As

pyridine, CH2Cl2, 25°C, 3 h,

Scheme 3: Synthesis of aminophenyl analogues. Reagents and conditions: a) Ac2O, pyri-
dine, 25°C, 3 h, 15: 82%; b) BzCl, Et3N, EtOAc, 0—25°C, 4 h, 17: 88%; 18: 90%; c) p-TsCl,
pyridine, CH2Cl2, 25°C, 20 h, 19: 92%.

Scheme 3.

Different substituents on the aryl ring could be incor-

corporated either after the Suzuki reaction or after the TBS deprotection step. We investigated different

hydrogen bond donor and acceptor groups in the ortho-, meta-, and para-positions of the aryl ring. As

a result of our initial docking studies, a series of car-

boxy- and amino-phenyl analogues were selected for

synthesised from the Suzuki coupling of methylcarboxyphenylbor-

onic acid. Subsequent hydrolysis under basic conditions fol-

lowed by amide coupling, using (benzotriazol-1-yloxy)tris(di-

methylamino)phosphonium hexafluorophosphate (BOP) in the

presence of N,N-diisopropylethylamine (DIPEA), was conducted

to form the amide analogues (Scheme 2). The ortho-substitut-

ed aryl analogues proved difficult to synthesise due to steric

hindrance, which resulted in little to no coupling. Analogue 28

was synthesised from the coupling of compound 55 and 2-meth-

oxycarbonylphenyl boronic acid, albeit in poor yield.

The target compounds for the aminophenyl series were syn-

thesised via acylation or sulfonylation of the aniline analogues

(scheme 2). Subsequent hydrolysis under basic conditions fol-

owed by amide coupling, using (benzotriazol-1-yloxy)tris(di-

methylamino)phosphonium hexafluorophosphate (BOP) in the

presence of N,N-diisopropylethylamine (DIPEA), was conducted

to form the amide analogues (Scheme 2). The ortho-substitut-

ed aryl analogues proved difficult to synthesise due to steric

hindrance, which resulted in little to no coupling. Analogue 28

was synthesised from the coupling of compound 55 and 2-meth-

oxycarbonylphenyl boronic acid, albeit in poor yield.

The target compounds for the aminophenyl series were syn-

thesised via acylation or sulfonylation of the aniline analogues

using acid chloride, anhydride, and sulfonyl chlorides

(Scheme 3). None of the desired cross-coupled product was

obtained from the Suzuki reaction of compound 6 and 2-amio-

nophenylboronic acid, therefore the ortho-substituted ana-

logues were not synthesised.

In addition to the carboxy- and amino-phenyl analogues, a

number of diverse boronic acids were used to investigate

small ring substituents; these boronic acids were coupled to

compound 6 affording analogues 47–54 after the removal of

the TBS group.

Biological Evaluation

Binding affinity to p38α MAPK

The synthesised analogues were evaluated using a fluorescence

polarisation (FP) binding assay developed by Munoz et al. using both nonphosphorylated and phosphorylated p38α en-

zymes. The fluoroprobe used was an analogue of the prototyp-

ical p38α inhibitor SB203580 attached to fluorescein. The Kd

value for the fluorescently labelled ligand was determined by

titrating against increasing concentrations of both the inactive

and active forms of p38α MAPK. The fluoroprobe was found to

have a Kd value of 13 nM to the inactive protein and 36 nM to

the active form of the protein. The binding affinities of com-

pounds 2, 3 and 28–55 are summarised in Table 1.

Most of the synthesised compounds showed little or no

binding to the nonphosphorylated protein, however a signifi-

cant improvement was observed in binding to the phosphory-

lated enzyme. ATP itself has a low affinity for the inactive p38α

enzyme relative to the active form. Since the synthesised ana-

logues are designed to in part mimic some of the interactions

ATP makes, namely the hydrogen bond to the Met109 back-

bone, the analogues are expected to behave similarly. One

crystal structure containing the dually phosphorylated p38α

MAPK protein exists in the protein databank. Figure 4 illus-

trates the differences between the apo nonphosphorylated

(PDB ID:1P38) and phosphorylated (PDB ID: 3PY3) p38α

MAPK crystal structures. Figure 4 shows that the phosphorylat-

ed p38α MAPK structure has a more exposed binding site indi-

cating that the conformational changes in the protein struc-

ture that occur as a result of phosphorylation cause the pro-

tein to open up in order to accommodate ATP. Therefore, the

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such as the benzydiamide or para-toluenesulfonamide group were attached to probe space. However these analogues did not bind to the protein. In most cases, substituents that were attached to the para position on the aromatic ring were generally poorer binders in comparison to the meta-substituted counterparts. Other analogues that demonstrated binding include 47, 50, and 53, which bear a meta-cyano, para-fluoro, and para-acetyl substituents, with \( K_i \) values of 1.8, 0.9, and 2.2 \( \mu M \), respectively. Synthesised intermediates were also evaluated in the binding assay. Interestingly, compound 2 was the best binder, which contained only the 4-fluorophenyl and 4-pyridyl rings attached to the thiophene. This compound had a \( K_i \) value of 0.6 \( \mu M \) demonstrating that the conversion to the thiophene core retains affinity to the p38\( \alpha \) protein.

Although several analogues displayed moderate affinity to phosphorylated p38\( \alpha \) MAPK, the tetra-substituted thiophenes were weaker binders compared to the pyridinyl imidazole RWJ67657 indicating that the proposed substituted phenyl moiety is not optimal in the pocket and further work is required to improve affinity.

### Cellular assays

In addition to the binding assay, the effects of the synthesised compounds on cardiac fibroblast collagen synthesis were investigated. Analogues with a \( K_i \) value of less than 2.2 \( \mu M \) to active p38\( \alpha \) MAPK were evaluated for the inhibition of rat neonatal cardiac fibroblast (NCF) collagen synthesis.\[10\] p38 MAPK is known to become activated in cardiac cells by a range of stimuli including angiotensin II (AngII)\[14,16\]. NCF collagen syn-

### Table 1. Binding affinities to inactive and active p38\( \alpha \) MAPK.

<table>
<thead>
<tr>
<th>Compd</th>
<th>( R^1 )</th>
<th>( R^2 )</th>
<th>( K_i ) [( \mu M )]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>inactive p38( \alpha )</td>
</tr>
<tr>
<td>RWJ67657</td>
<td></td>
<td></td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td></td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td></td>
<td>&gt;10</td>
</tr>
<tr>
<td>28</td>
<td>2-(CO(_2)Me)Ph</td>
<td>&gt;10</td>
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<td>29</td>
<td>3-(CO(_2)Me)Ph</td>
<td>&gt;10</td>
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<td>4-(CO(_2)Me)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>31</td>
<td>3-(CO(_2)H)Ph</td>
<td>&gt;10</td>
<td>7.1</td>
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<tr>
<td>32</td>
<td>4-(CO(_2)H)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>33</td>
<td>3-(CH(_2)OH)Ph</td>
<td>&gt;10</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>34</td>
<td>4-(CH(_2)OH)Ph</td>
<td>&gt;10</td>
<td>3.5</td>
</tr>
<tr>
<td>35</td>
<td>3-(CON(_2)H)Ph</td>
<td>&gt;10</td>
<td>2.2 ± 0.3</td>
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<tr>
<td>36</td>
<td>4-(CON(_2)H)Ph</td>
<td>&gt;10</td>
<td>2.1 ± 1</td>
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<td>37</td>
<td>3-(CONH(_2))(CH(_2))OH</td>
<td>&gt;10</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>38</td>
<td>4-(CONH(_2))(CH(_2))OH</td>
<td>&gt;10</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>39</td>
<td>3-(NH(_2))</td>
<td>&gt;10</td>
<td>3.2</td>
</tr>
<tr>
<td>40</td>
<td>4-(NH(_2))</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>41</td>
<td>3-(NHAc)Ph</td>
<td>2.6 ± 0.6</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>42</td>
<td>4-(NHAc)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>43</td>
<td>3-(NH(_2)N)Ph</td>
<td>&gt;10</td>
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<td>44</td>
<td>4-(NH(_2)N)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>45</td>
<td>3-(NHSO(_2))toluene)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td>46</td>
<td>4-(NHSO(_2))toluene)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>47</td>
<td>3-(CN)Ph</td>
<td>&gt;10</td>
<td>1.8 ± 0.4</td>
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<tr>
<td>48</td>
<td>3-(Me)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>49</td>
<td>4-(Me)Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>50</td>
<td>4-F-Ph</td>
<td>&gt;10</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>51</td>
<td>4-Cl-Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>52</td>
<td>4-(CF(_3))Ph</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td>53</td>
<td>4-(Ac)Ph</td>
<td>&gt;10</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>54</td>
<td>4-Oh-Ph</td>
<td>&gt;10</td>
<td>3.4</td>
</tr>
<tr>
<td>55</td>
<td>I</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

Experiments were performed in duplicate with \( n = 2-3 \) for compounds with \( K_i \) values: [a] \(< 10 \mu M \) to inactive p38\( \alpha \) and [b] \(< 3 \mu M \) to active p38\( \alpha \). All other compounds were screened in duplicate with \( n = 1 \).
thesis determined by \(^3\)H-proline incorporation, is upregulated in response to AngII stimulation (Figure 5). The cardiac fibroblasts were pre-treated with the synthesised analogues at 0.1, 1, 3, and 10 µm concentrations followed by stimulation with AngII (Figure 5). All of the tested analogues suppressed AngII-stimulated collagen synthesis at 3 and 10 µm concentrations. Few compounds however, inhibited collagen synthesis at 1 µm concentration consistent with the weak binding of the analogues observed in the fluorescence polarisation assay. Compound 53 shows stronger inhibition of collagen synthesis but this may be attributed to other interactions within the cell.

**Conclusions**

A number of substituted thiophenes were synthesised and shown to bind with higher affinity to the phosphorylated active form of p38 MAPK relative to the nonphosphorylated inactive form. This work shows successful modification of the core to a thiophene with compound 2 having a \(K_i\) value of 0.6 µm to the active enzyme. The tetra-substituted thiophene series showed modest inhibition of p38\(\alpha\) MAPK with binding affinities in the low micromolar range. These compounds also demonstrate cellular activity; however, further work is required to optimise interactions within the binding pocket and generate more potent compounds. The fact that compound 2, bearing only the 4-fluorophenyl and 4-pyridyl substituents, displayed the highest affinity suggests that the additional alkyne and aryl substituents are not necessary for binding. Therefore, further alterations to these substituents must be investigated in order to determine whether any stronger or more favourable interactions can be achieved. Optimisation of these substituted thiophenes may lead to p38 MAPK inhibitors that can be used in the treatment of cardiac disease.

**Experimental Section**

**Molecular Modelling–Ensemble Docking**

In our previous work we identified the 1BL7–2EWA ensemble as a good model for docking p38\(\alpha\) MAPK inhibitors containing the 4-fluorophenyl and 4-pyridyl ring substituents.\(^{[15]}\) Therefore, this model was used to dock designed ligands.

The designed ligands suggested for p38\(\alpha\) MAPK inhibition were built in ChemBioDraw Ultra 12.0 (CambridgeSoft) and prepared using LigPrep v2.4 (Schrödinger) to convert the two-dimensional structures to their respective three-dimensional structures. The 1BL7\(^{[7a]}\) and 2EWA\(^{[20]}\) crystal structures were downloaded from the Protein Data Bank (PDB)\(^{[21]}\) and prepared using the Protein Preparation Wizard in Maestro v9.2 (Schrödinger) for the addition of hydrogens and assignment of bond orders and partial charges. Prime v2.2 (Schrödinger) added side chains to residues with missing atoms. Each protein was refined using exhaustive sampling and minimisations were conducted only on the newly added hydrogens.

Docking into both the 1BL7 and 2EWA crystal structures was conducted using the Glide v5.6 (Schrödinger) extra precision (XP) method. For the ensemble analysis, the glidescores of best conformation of each ligand were taken from each crystal structure and averaged. The compounds were re-ranked based on the ensemble score (see Supporting Information).

**Chemistry**

**General remarks**: All chemical reagents were acquired from Sigma–Aldrich, Fluka, Merck, Boron Molecular, and Matrix Scientific and were used without further purification. Flash chromatography was carried out using Scharfau silica gel 60, 0.06–0.20 mm (70–230 mesh ASTM). Melting points were determined using a Mettler Toledo MP50 melting point apparatus. NMR spectra were recorded on a 300 MHz Bruker Avance DPX 300 NMR spectrometer.
a 400 MHz Bruker Avance Ultrashield Plus NMR spectrometer, or a 600 MHz Varian Unity Inova NMR spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) referenced to an internal standard of residual protio- solvent ([1]H NMR, [1]C NMR: CDCl₃, (7.26, 77.16), CD₂OD (3.31, 49.0) or [2]D-DMSO (2.50, 39.52).[22] Multiplicity is quoted as app. (apparent), br. (broad), s (singlet), d (doublet), t (triplet), q (quartet), p (pentet) and m (multiplet). Coupling constants (J) are given in Hertz (Hz). Low resolution mass spectrometry (LRMS) analyses were performed using a Micromass Platform II single quadrupole mass spectrometer equipped with an atmospheric pressure (ESI/APCI) ion source. Sample management was facilitated by an Agilent 1100 series high performance liquid chromatography (HPLC) system using MassLynx version 3.5 software. High resolution mass spectrometry (HRMS) analyses were carried out on a Waters Micromass LCT Premier XE Orthogonal Acceleration time-of-flight (TOF) mass spectrometer coupled to an Alliance 2795 Separation Module using MassLynx version 4.1 software. Liquid chromatography–mass spectrometry (LCMS) was performed on an Agilent 1200 Series Separation Module fitted with a 6.120 quadrupole detector and a Phenomenex Luna C8(2) 100Å column (50×4.6 mm, internal diameter) 5 μm column. Samples were run in a gradient of 5–100% buffer B in buffer A (buffer A: 0.1% acq formic acid; buffer B: 80% CH₃CN, 19.9% water, 0.1% tri-fluoroacetic acid) over 10 min, followed by isocratic 100% buffer B for 1 min then a gradient of 100–20% buffer B over 1 min followed by a flow rate of 0.5 mL/min. Agilent Chemstation software (version 8.04.01) managed the running and processing of samples. Analytical reversed phase (RP) HPLC was acquired on a Waters Millennium 2690 system fitted with a Phenomenon Luna C8 100Å (50×4.6 mm, internal diameter) 5 μm column with UV detection at 254 nm. Samples were run in a gradient of 20–100% buffer B in buffer A (buffer A: 0.1% acq trifluoroacetic acid; buffer B: 80% CH₃CN, 19.9% water, 0.1% tri-fluoroacetic acid) over 10 min, followed by isocratic 100% buffer B for 1 min then a gradient of 100–20% buffer B over 1 min followed by isocratic 20% buffer B for 10 min at a flow rate of 1.0 mL/minute. EmpowerPro managed the running and processing of samples. Microwave chemistry was performed using a Biotage Initiator Microwave Reactor according to manufacturer’s instructions.

4-2-(4-Fluorophenyl)thiophen-3-yl)pyridine (2): To a solution of 2.3-dibromothiophene (2.38 mL, 20.7 mmol) in AcOH (18 mL) were added Hg(OAc)₂ (3.23 g, 10.1 mmol). The solution was heated at 70°C for 16 h. Concurrently, I₂ (5.14 g, 20.2 mmol) and KI (3.36 g, 20.2 mmol) were dissolved in H₂O (38 mL) over 16 h in a separate round-bottom flask. After 16 h, the AcOH mixture was concentrated in vacuo and poured into ice water (100 mL). The resulting white precipitate was filtered and washed with H₂O then Et₂O to afford the mercuric acetate intermediate as a white powder. The intermediate was added to the KI solution. THF (1 mL) was added to break the surface tension, and the mixture was stirred at 25°C for 16 h. Saturated Na₂S₂O₃ (100 mL) was added, and the resulting yellow solid was filtered and washed with H₂O (50 mL). The solid was dissolved in THF/EtOAc (100 mL) and washed further with saturated Na₂S₂O₃ (3×50 mL). The organic extract was dried over MgSO₄, filtered, and concentrated in vacuo to afford thioephene 3.

Recrystallisation from THF/EtOAc (1:1) afforded compound 3 (C₁₅H₁₀FNS: M⁺ = 507.10) as yellow crystals (1.39 g, 81%); mp 212.8°C (decomp); [1]H NMR (400 MHz, [2]D-DMSO): δ = 8.59–8.56 (m, 2H), 7.20–7.12 ppm (m, 6H); [1]C NMR (101 MHz, [2]D-DMSO): δ = 162.0 (d, JCF = 247.0 Hz), 149.7, 145.1, 145.0, 140.1, 130.9 (d, 2JCF = 8.5 Hz), 128.4 (d, 2JCF = 3.2 Hz), 125.5, 115.8 (d, 2JCF = 21.9 Hz), 101.8, 87.6 ppm; HRMS-ToF-ESI: m/z [M + H]⁺ calcd for C₁₅H₁₁FN₅S: 507.8524, found 507.8521; LCMS (ESI): tR = 6.4 min, 507.9 [M + H]⁺; RP-HPLC: tR = 8.4 min, >99%.

(But-3-yn-1-ol)(tert-butyl)dimethylsilane (5):[25] Compound 5 was synthesised using a similar procedure by Nadeau et al.[22] To a solution of 3-butyn-1-ol (3.00 g, 42.8 mmol) in CH₂Cl₂ (60 mL) was added imidazole (7.28 g, 107 mmol) and cooled to 5°C. TBSCl (6.45 g, 42.8 mmol) was added and the reaction mixture was stirred at 25°C for 16 h. CH₂Cl₂ (100 mL) was added and the mixture was washed with H₂O (2×50 mL) and brine (50 mL). The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo to afford compound 5 (7.73 g, 98%) as a colourless oil. 5: C₃H₇O₂Si (M⁺ = 184.35); [1]H NMR (400 MHz, CDCl₃): δ = 3.74 (t, J = 7.1 Hz, 2H), 2.40 (td, J = 7.1, 2.7 Hz, 2H), 1.95 (t, J = 2.7 Hz, 1H), 0.90 (s, 9H), 0.07 (s, 6H); [1]C NMR (101 MHz, CDCl₃): δ = 81.6, 69.4, 61.9, 26.0, 23.0, 18.4, −5.2 ppm.

4-(5-(4-Fluorobutyl)dimethylsilyloxy)-but-1-yn-1-yl)-4-iodothiophen-3-yl)pyridine (6): Negishi coupling: To a solution of allyne 5 (2.06 mL, 9.97 mmol) in THF (15 mL) was added dropwise nBuLi (1.2 M, 8.3 mL, 10 mmol) at 0°C. The reaction mixture was stirred for 15 min. ZnCl₂ (1.63 g, 12.0 mmol) was added, and the reaction mixture was stirred at 0°C for 15 min, then allowed to warm to 25°C for 15 min, at which time the zinc had dissolved. Concurrently, compound 3 (1.23 g, 2.43 mmol) was dissolved in THF (18 mL), and nitrogen was bubbled through the solution for 30 min. The metallated alkyne solution was added dropwise to the thiophene solution followed by addition of tetrakis(triphenylphosphine)palladium(0) (0.283 g, 0.245 mm). The mixture was stirred at 25°C for 70 h. Saturated NH₄Cl (7.5 mL) was added, and the mixture was stirred for 15 min. EtOAc (150 mL) was added and the mixture was washed with saturated Na₂CO₃ (3×40 mL) and brine (3×40 mL). The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo. The product was purified by column chromatography using a gradient elution (0–50% EtOAc/petroleum spirits) to afford a pale yellow solid. Recrystallisation from Et₂O gave thiophene 2 (C₁₅H₁₀FNS· M = 255.31) as a white powder (3.81 g, 71%): mp 95.8–97.6°C; [1]H NMR (400 MHz, CDCl₃): δ = 8.51 (br. app. d, J = 5.6 Hz, 2H), 7.37 (d, J = 5.2 Hz, 1H), 7.28–7.23 (m, 2H), 7.18 (d, J = 5.3 Hz, 1H), 7.16–7.15 (m, 2H), 7.04–6.98 ppm (2H); [1]C NMR (101 MHz, CDCl₃): δ = 162.3 (d, JCF = 248.3 Hz), 149.8, 143.6, 139.7, 130.9 (d, JCF = 8.1 Hz), 129.33 (d, JCF = 3.4 Hz), 129.27, 125.0, 123.4, 115.6 ppm (d, JCF = 21.7 Hz); HRMS-ToF-ESI: m/z [M + H]⁺ calcd for C₁₅H₁₁FN₅S: 507.0691, found 506.0589; LCMS (ESI): tR = 4.9 min, 256.1 [M + H]⁺; RP-HPLC: tR = 6.5 min, 99%.

ChemistryOpen 2015, 4, 56 – 64 62
(0.093 g, 0.132 mmol). The reaction mixture was bubbled with nitrogen for 15 min and heated at 120 °C for 2 h. The reaction mixture was diluted with EtOAc (100 mL) and washed with H2O (3 × 50 mL) and brine (50 mL), dried over MgSO4, filtered and concentrated in vacuo. The resulting oil was purified by column chromatography using a gradient elution (0–50% EtOAc/petroleum spirits) to afford compound 6 as a white solid. Trituration with petroleum spirits gave compound 6 (C74H75FNO3Si: M = 563.54) as a white powder (11.9 g, 82%): mp 96.6–97.7 °C; 1H NMR (400 MHz, CDCl3); δ = 8.60 (br. app. d, J = 4.8 Hz, 2H), 7.13–7.11 (m, 2H), 7.10–7.05 (m, 2H), 6.94–6.89 (m, 2H), 3.87 (t, J = 6.9 Hz, 2H), 2.73 (t, J = 6.9 Hz, 2H), 0.92 (s, 9H), 0.11 ppm (s, 6H); 13C NMR (101 MHz, CDCl3); δ = 162.8 (d, 1JCF = 249.8 Hz), 150.1, 144.9, 141.0, 138.3, 130.9 (d, 1JCF = 8.3 Hz), 128.6 (d, 1JCF = 3.4 Hz), 125.7, 115.9 (d, 1JCF = 21.9 Hz), 96.9, 91.8, 75.7, 61.6, 21.6, 24.5, 18.5, –5.1 ppm (n.b. Missing a quaternary carbon resonance in the 13C NMR); HRMS-ToF-ESI: [M + H]+ calcd for C25H28FINOSSi8; RP-HPLC: tR = 7.8 min, 564.1 [M + H]+; RP-HPLC: tR = 10.9 min, 97%.

General procedure for Suzuki coupling: To a solution of thio- phene 6 (100 mg, 1.0 equiv) in THF (3 mL) was added the boronic acid/pinacol ester (3.0 equiv) and Na2CO3 (1.19 g, 11.9 mmol). Purification using gradient column chromatography (20–50% EtOAc/petroleum spirit) gave compound 8 (C74H75FNO3Si: M = 571.78) as a yellow solid (226 mg, 89%): 1H NMR (400 MHz, CDCl3); δ = 8.38–8.36 (m, 2H), 7.96–7.95 (m, 1H), 7.94–7.91 (m, 1H), 7.29 (app. td, J = 7.7, 5.7 Hz, 2H), 7.23–7.21 (m, 1H), 7.15–7.10 (m, 2H), 6.98–6.92 (m, 2H), 6.83–6.82 (m, 2H), 3.87 (s, 3H), 3.70 (t, J = 7.1 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 0.87 (s, 9H), 0.04 ppm (s, 6H); LCMS (ESI): tR = 7.6 min, 572.3 [M + H]+.

Methyl 3-(2-(4-((tert-butyl(dimethyl)silyl)oxy)but-1-yn-1-yl)-4-methylphenyl)phenoxy)phenylboronic acid (45): Compound 8 was synthesised using the general method for Suzuki coupling from thioephene 6 (250 mg, 0.444 mmol) and 3-(4-methoxy- carbonyl)phenylboronic acid (240 mg, 1.33 mmol). Purification using gradient column chromatography (20–50% EtOAc/petroleum spirit) gave compound 8 (C74H75FNO3Si: M = 571.78) as a yellow solid (226 mg, 89%): 1H NMR (400 MHz, CDCl3); δ = 8.38–8.36 (m, 2H), 7.96–7.95 (m, 1H), 7.94–7.91 (m, 1H), 7.29 (app. td, J = 7.7, 5.7 Hz, 2H), 7.23–7.21 (m, 1H), 7.15–7.10 (m, 2H), 6.98–6.92 (m, 2H), 6.83–6.82 (m, 2H), 3.87 (s, 3H), 3.70 (t, J = 7.1 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 0.87 (s, 9H), 0.04 ppm (s, 6H); LCMS (ESI): tR = 7.6 min, 572.3 [M + H]+.

Neonatal cardiac fibroblast (NCF) culture: The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (PHS Approved Animal Welfare Assurance #A5587–01). All animal usage was also approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (AEC) in accordance with National Health and Medical Research Council (NHMRC) guide for the care and use of laboratory animals (AEC # E/09/01/2010M). Sprague–Dawley rat neonatal cardiac fibroblasts (NCFs) were isolated from one- to two-day-old pups with enzymatic digestion as described in detail previously.[16,24] NCFs were seeded and maintained in high-glucose (25 mm) Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Mount Waverley, Australia) in the presence of 1% antibiotic/antimycotic (Invitrogen, Mount Waverley, Australia) and 10% foetal bovine serum (JRH biosciences, Lenexa, USA). NCFs were used at passage two.[16,24] NCF collagen synthesis assay: NCF collagen synthesis was determined by 3H-proline incorporation as described previously.[16,24] NCFs were seeded at a density of 50,000 cells/well in 12-well plates and incubated (37 °C, 5% CO2) overnight before serum starvation with 0.5% bovine serum albumin (BSA) for 48 h. Cells were then pre-treated in the presence or absence of test compounds (0.1, 1,
3, or 10 μM) for 2 h before stimulation with 100 nM AngII in the presence of 0.5% BSA and addition of 1 μCi of ‘H-proline to each well. After 48 h of further incubation, cells were harvested by 10% trichloroacetic acid (TCA) preparation, and ‘H-proline incorporation was determined as previously described.[26–27]"