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The effect of RO3201195 and a pyrazolyl ketone P38 MAPK inhibitor library on the proliferation of Werner syndrome cells†

Mark C. Bagley,*a Jessica E. Dwyer,a Mohammed Baashen,†a Matthew C. Dix,b Paola G. S. Murziani,b Michal J. Rokicki,c David Kiplingc and Terence Davis*c

Microwave-assisted synthesis of the pyrazolyl ketone p38 MAPK inhibitor RO3201195 in 7 steps and 15% overall yield, and the comparison of its effect upon the proliferation of Werner Syndrome cells with a library of pyrazolyl ketones, strengthens the evidence that p38 MAPK inhibition plays a critical role in modulating premature cellular senescence in this progeroid syndrome and the reversal of accelerated ageing observed in vitro on treatment with SB203580.

Introduction

P38α is one isoform of the mitogen-activated protein kinase (MAPK) intracellular enzymes which are central to the regulation of cytokine biosynthesis and inflammatory cell signalling.1,2 The reduction of pro-inflammatory cytokine levels offers a means for the treatment of inflammatory disorders such as rheumatoid arthritis and atherosclerosis.3,4 In addition to inflammatory disorders, p38α is known to play a role in the resistance of various cancers to chemotherapy, cancer proliferation and metastasis.5,6 This makes p38α a compelling therapeutic target for the design of safe and efficacious inhibitors suitable for clinical investigation.2,7 Following the discovery that pyridinylimidazole p38α inhibitors such as SB203580 mediate multiple cellular responses,1 including the production of inflammatory cytokines, a wide variety of structurally-distinct chemotypes8,9 have been discovered to inhibit this enzyme with notable differences in binding motif.10 Many of these have been used in stage II or III clinical trials.11

Werner syndrome (WS) is a rare autosomal recessive disorder.12 The mutated gene (WRN) encodes for a RecQ helicase involved in DNA replication, recombination and repair. Individuals living with the syndrome display the premature onset of many of the clinical features of old age, show early susceptibility to a number of major age-related diseases and have an abbreviated median life expectancy (47 years). Consequently, WS is widely used as a model disease to investigate the mechanisms underlying normal human ageing. Associated with these aged features, fibroblasts from WS individuals have a much-reduced replicative capacity when compared with fibroblasts from normal individuals, and have a stressed, aged, morphology and high levels of activated p38α.13–15 This accelerated cellular senescence is thought to underlie whole body ageing in this syndrome. As part of our interest in mechanisms of premature cell senescence, we showed that administering the p38α inhibitor SB203580 to WS cells rescues all of the features of accelerated replicative decline.13 This observation suggested that the abbreviated life span of WS cells is due to stress-induced growth arrest mediated by p38α MAPK, which we speculate is transduced from the frequently stalled DNA replication forks observed in these cells.16 It also offers a means to clinically regulate this process and, by therapeutic means, intervene in a premature ageing syndrome.16

However, it has been shown that SB203580 is not specific for p38α, but also inhibits the related N-terminal c-Jun kinases (JNKs) that may play a role in cellular senescence.17–19 Thus more work is needed using inhibitors that do not target the JNKs to verify the role played by p38α in WS cells. In addition, SB203580 is not suitable for possible in vivo use.20

This manuscript describes a rapid route to one such inhibitor, RO3201195 (1),20 the study of this inhibitor in WS cells, and its comparison with the canonical p38α inhibitor SB203580 to investigate the role of p38α MAPK signal transduction in replicative senescence. This pyrazolyl ketone p38α MAPK inhibitor progressed to clinical trials, is orally bioavailable, exhibits high kinase selectivity, and inhibited the...
production of the cytokines IL-6 and TNFα upon lipopolysaccharide (LPS) challenge in rats.20 Thus, RO3201195 could be a valuable chemical tool in dissecting the accelerated ageing pathophysiology seen in WS cells and its rescue in vitro using small molecule inhibitors of this stress signalling pathway.13,21 Our rapid approach to the inhibitor employs microwave irradiation to accelerate a number of steps, preparing the central pyrazole motif through cyclocondensation of hydrazine 3 and a suitably protected benzoylnitril 2, derivatized by homologation prior to heterocycle formation (Fig. 1). This approach had been successful for the synthesis of a pyrazolyl ketone library (4)22 and so should be suitable for rapid production of the target inhibitor under microwave-assisted conditions.

Results and discussion

The synthesis of a suitably functionalized β-ketonitrile derivative 2, as a precursor for pyrazole formation, was first investigated using the Pd-catalyzed Heck reaction23,24 of 3-iodoanisole and 3-methoxyacrylonitrile (5). This approach had the advantage that, potentially, it could access 3-methoxybenzoylacetonitrile (6) directly from commercially-available materials, upon acid hydrolysis of the coupled product, methyl vinyl ether 7 (Scheme 1, top), providing a very rapid route into the pyrazole skeleton. Heck reactions of α- or β-substituted enol ethers are known and have been shown to provide efficient access to 1-arylpropanone derivatives.25 However, although there are numerous examples of Heck reactions of α,β-disubstituted alkenes such as methacrylates,26 3-ethoxyacrylates,27 cinnamates,28 crotonates29 and alkoxypropenes,30 there are very few examples of β-alkoxyacrylonitriles in these and related processes. Enol ethers are less reactive than electron-poor alkenes in Heck transformations and so these slow reactions often require high catalyst loadings, more reactive aryl iodides as substrates and the use of certain additives, particularly when performed under solid–liquid phase transfer conditions.31 Furthermore, α,β-disubstituted alkenes may suffer from steric constraints and so, although direct, this approach for the synthesis of benzoylacetonitriles was untested. Ortar et al.32 did describe the arylation of an α-methoxyacrylate back in 1987 using aryl iodides, palladium acetate (3 mol%), NaHCO3 base and tetrabutylammonium chloride as additive. Using Jeffery’s conditions for Heck-type processes as a starting point,33 methods related to the conditions for arylation of acrylonitrile with aryl iodides34 were investigated in the Heck reaction of 3-iodoanisole and 3-methoxyacrylonitrile (5). The use of Pd(OAc)2 (10 mol%) as catalyst in MeCN–H2O (3 : 1) (Table 1) under microwave-assisted heating at 130 °C for 1.5 h (entry 4) with K2CO3 as base and tetrabutylammonium bromide (TBAB) as additive gave, following aqueous work up, the α-aryl enol ether 7 as a mixture of (E)- and (Z)-diastereoisomers, in a 2 : 1 ratio, as determined by 1H NMR spectroscopic analysis. The diastereoisomers could be separated by column chromatography, albeit in variable and poor yield. Low conversions and poor diastereoccontrol has been observed before in Heck reactions of β-alkoxyacrylonitriles25 but, in our case, separation would not be required if the mixture could be converted directly to the target benzoylacetonitrile 6.

Surprisingly the hydrolysis of the enol ether diastereomeric mixture 7 required very forcing conditions. Simple treatment of the Heck reaction mixture with acid under ambient conditions failed to provide β-ketonitrile 6. The hydrolysis of related alkoxyacrylonitriles were notable, by their absence,
from the study of Doucet and Santelli following Heck reaction of 3-iodoanisole, catalyzed by a tetraphosphane/palladium complex. However, by resorting to more forcing conditions, gratifyingly, microwave irradiation of the isolated diastereoisomeric mixture 7 in CHCl–MeOH (1:4) at 110 °C for 30 min finally provided benzoylacetonitrile 6 after purification by column chromatography but only in modest isolated yield over the two steps (Table 1, entry 1, 36%).

Table 1 Investigating conditions for Heck reaction of 3-iodoanisole and 3-methoxyacrylonitrile (5) to give benzoylacetonitrile 6

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Additive</th>
<th>Conditions</th>
<th>Yielda (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K₂CO₃</td>
<td>TAB</td>
<td>Conductive heating, 90 °C, 24 h</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>TAB</td>
<td>Conductive heating, 90 °C, 24 h</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>K₂CO₃</td>
<td>TAB</td>
<td>Microwaves, 130 °C, 1 h</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>K₂CO₃</td>
<td>TAB</td>
<td>Microwaves, 130 °C, 1.5 h</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>K₂CO₃</td>
<td>TAB</td>
<td>Microwaves, 140 °C, 1 h</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>K₂CO₃</td>
<td>TAB</td>
<td>Microwaves, 150 °C, 1.5 h</td>
<td>31</td>
</tr>
<tr>
<td>7</td>
<td>K₂CO₃</td>
<td>DPPP</td>
<td>Microwaves, 150 °C, 1.5 h</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 2 Investigating conditions for base-mediated Claisen condensation of methyl benzoate 9 and acetonitrile to give benzoylacetonitrile 6

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Conditions</th>
<th>Yielda (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaOEt</td>
<td>MeCN, RT; reflux, 24 h</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>NaOMe</td>
<td>MeCN, RT; reflux, 24 h</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>NaH</td>
<td>THF, reflux, 1 h</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>NaH</td>
<td>THF, microwaves, 150 °C, 1 h</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>NaH</td>
<td>PhMe, 90 °C, 16 h</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>LDA</td>
<td>THF, −78 °C, 1.5 h</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>LDA</td>
<td>THF, −50 °C, 3 h</td>
<td>70</td>
</tr>
</tbody>
</table>

A well-established method for Claisen condensation using sodium ethoxide or sodium methoxide as base was frustrated by the almost immediate formation of a homogenous gelatinous mass and so a range of conditions were investigated (Table 2). Heating this mixture in acetonitrile at reflux for 24 h gave only a very low yield (9% in both cases) of the benzoylacetonitrile 6 (entries 1 and 2) and significant return of unreacted ester and so alternative methods were employed. Carrying out the reaction using NaH in THF at reflux for 1.5 h gave an immediate improvement in efficiency and provided the condensation product 6 in 15% yield (entry 3), 35% yield if heated at 150 °C for 1 h in a sealed tube under microwave irradiation (entry 4). Changing the solvent to toluene and heating the mixture at 90 °C for 16 h gave a similar yield (entry 5, 34%). However, the use of freshly-prepared LDA as base at −50 °C in THF (entry 7) gave the Claisen condensation product 6 in good yield (70%), whereas at lower temperatures was inefficient (entry 6). Given the high yield and speed of the microwave-assisted esterification, this route now represented the quickest and most efficient means to access this key intermediate.

Considering our previous success in the use of microwave irradiation in cyclocondensation reactions for the preparation of pyrazoles, the aminopyrazole core motif of BIRB 796 and pyrazolyl ketone libraries, was homologated in a microwave-assisted Knoevenagel condensation using N,N′-diphenylformamidine (10) in xylenes at 180 °C for 20 min to give enaminoine 11 in good yield (74%) (Scheme 2). Cyclocondensation by microwave irradiation with the hydrochloride salt of 4-fluorophenylhydrazine (3) in the presence of Et₃N in EtOH at 140 °C for 1 h gave 5-aminopyrazole 12 in superior yield (86%) to a range of other methods, after purification by column chromatography. Protodemethylation using BBr₃ in CH₂Cl₂ (1 M) at RT overnight gave the corresponding phenol 13 in quantitative yield, which was reacted in a S_N2 displacement reaction with ketal-protected tosyl glycerate 14 under basic conditions. The reaction in DMF in the presence of K₂CO₃ at 80 °C for 24 h gave only a very low yield of product 15 (9%) but switching to DMSO and carrying
out the $S_N$2 displacement at a slightly higher temperature (100 °C) for prolonged period (40 h) gave phenyl ether 15 in improved yield (42%) after purification by column chromatography, followed by recrystallization. Submitting the product of tosylate displacement 15 to ketal deprotection directly, prior to recrystallization, under acid-catalyzed aqueous conditions at 50 °C for 18 h gave RO3201195 (1) in 37% yield over the two steps, after purification by column chromatography and recrystallization. The target inhibitor exhibited spectroscopic data that matched literature values.

Scheme 2 Microwave-assisted synthesis of RO3201195. Reagents and conditions: (i) xylene, microwaves, 180 °C, 20 min; (ii) Et$_3$N, EtOH, 140 °C, 1 h; (iii) BBr$_3$in CH$_2$Cl$_2$ (1 M), 0 °C-RT, 18 h; (iv) K$_2$CO$_3$, DMSO, 100 °C, 40 h; (v) TsOH, MeOH–H$_2$O, 50 °C, 18 h.

Due to limited supplies of primary WS fibroblasts, initial experiments to determine the effects of novel kinase inhibitors on cellular proliferation were performed using WS$^{\text{tert}}$ cells (see materials and methods for a description of these cells). Despite being immortal, these telomerized cells retain the slow proliferation rate typical of primary WS cells and show a similar response to SB203580 treatment. Thus, these cells can be used to test the effects of modulators of the p38-signalling pathway prior to their use on primary cells.

WS$^{\text{tert}}$ cells were grown in standard medium supplemented with SB203580 at a final concentration ranging from 10 nM to 30 µM (Fig. 2a). SB203580 inhibited p38α even at the lowest doses used of 10 nM with maximum inhibition seen at 1.0 µM and above (Fig. 2c), and has an IC$_{50}$ for p38α of approximately 30 nM as measured by us, and others. SB203580 treatment resulted in an increased proliferation rate of the WS$^{\text{tert}}$ cells compared to DMSO-treated control cells even at the lowest con-
concentration used, with the effect on proliferation rate increasing steadily with increasing SB203580 concentration and reaching a maximum between 2.5 µM and 10 µM. When the level of p38α activity was plotted against the effects upon proliferation rate a sigmoid curve was found (Fig. 2e). At 2.5 µM SB203580 resulted in an increase in proliferation rate of approximately 22% compared to DMSO controls (Fig. 2a and e).

RO3201195 has an IC_{50} of approximately 190 nM with maximal inhibition between 2.5 and 10 µM. In this work, RO3201195 inhibited p38α at a minimum concentration of 25 nM, with the maximal effect seen at 10.0 µM (Fig. 2d). RO3201195 had an increasing effect on the proliferation rate of WS\textsuperscript{tert} cells up to 10.0 µM concentration, thereafter becoming inhibitory (Fig. 2b). As seen for SB203580, when the level of p38α activity was plotted against the effects of RO3201195 upon proliferation rate a sigmoid curve was found (Fig. 2e): indeed the curve was essentially identical with the curve obtained using SB203580. The maximal increase seen in the proliferation rate of WS\textsuperscript{tert} cells was 19% at 10.0 µM (Fig. 2b and e).

The above data show that the optimal concentration of these inhibitors for the maximal effects on WS\textsuperscript{tert} cells are 2.5 µM for SB203580 and 10.0 µM for RO3201195. The inhibitors completely inhibited p38α but did not inhibit total JNK activity in WS\textsuperscript{tert} cells at these concentrations as measured by analyzing the phosphorylation of their downstream targets HSP27 and c-Jun by immunoblot (Fig. 2h). WS\textsuperscript{tert} cells are stimulated with anisomycin that activates p38α and JNKs as shown by the increased presence of the band in the A lane of the p-HSP27 panel, and the appearance of the doublet in the p-c-Jun and c-Jun panels (Fig. 2h). RO3201195 at 10.0 µM and SB203580 at 2.5 µM concentration reduced the p-HSP27 band to the level seen in the unstimulated control (C lane), but had no effect on the doublet seen in p-c-Jun and c-Jun.

We next used the p38α inhibitors on the primary Werner syndrome fibroblast strain WAG05229 that was close to replicative senescence to determine their effects on cellular senescence. For SB203580 a concentration of 2.5 µM was used, whereas for RO3201195 10.0 µM concentration was employed, as these were the lowest concentrations that resulted in maximal proliferation rate and 100% p38α inhibition (see Fig. 2). The cells were re-fed with DMEM containing the inhibitors as described previously.\textsuperscript{15} DMSO-treated fibroblasts proliferated very slowly and only achieved a single population doubling in the 88 days of the experiment (Fig. 2f). Inhibitor treated cells, however, had a much elevated proliferation rate, and managed approximately 7.5 population doublings prior to growth arrest (Fig. 2f). There was essentially no difference whichever inhibitor was used. Thus, as the effects of these inhibitors on WS cells are essentially identical and yet they show very different kinase specificity profiles at the concentrations used (the only common denominator being p38), the conclusion is that the effects on proliferation seen in both WS\textsuperscript{tert} cells and primary WS fibroblasts result from the inhibition of p38α.

Finally, we used a pyrazolyl ketone library 4 (Table 3) of compounds related to RO3201195\textsuperscript{22} for their effects on the proliferation of WS\textsuperscript{tert} cells (Fig. 2g). These were used at a concentration of 1.5 µM that resulted in a varied degree of p38α inhibition from approximately 5% to greater than 95% (Fig. 2g and ref. 22). When the effect on the proliferation rate of WS\textsuperscript{tert} cells by this pyrazolyl ketone library were plotted against the % p38α activity (as assessed by ELISA\textsuperscript{22}), a linear response was seen (Fig. 2g), providing further support that these effects on cellular proliferation are due to p38α inhibition.

The data presented in this paper showing that RO3201195 has essentially identical effects on the proliferation of WS cells as SB203580, and that the proliferation rate is dependent on p38 activity, provide very strong support that the prevention of premature senescence seen in WS fibroblasts by SB203580 reported previously\textsuperscript{13} is via p38α signalling. If premature cellular senescence does underlie the accelerated ageing seen in WS individuals, these data also provide further support that the use of p38 inhibitors in vivo may alleviate this accelerated ageing.\textsuperscript{16} Finally, RO3201195 is shown to be a useful tool for the dissection of signalling pathways in human cells, as it is efficacious, relatively potent and is more kinase selective than SB203580.\textsuperscript{20} In addition, it has shown therapeutic efficacy in vivo in rats in regulating cytokine production upon LPS challenge,\textsuperscript{20} so may prove useful for future in vivo use.

### Experimental

#### Materials and methods

Commercially available reagents were used without further purification; solvents were dried by standard procedures. Light petroleum refers to the fraction with bp 40–60 °C and ether refers to diethyl ether. Unless otherwise stated, reactions were performed under an atmosphere of air. Flash chromatography was carried out using Merck Kieselgel 60 H silica or Matrex silica 60. Analytical thin layer chromatography was carried out using aluminium-backed plates coated with Merck Kieselgel.

| Table 3 Pyrazolyl ketone library 4 studied in WS\textsuperscript{tert} cells\textsuperscript{a} |
|---|---|
| **R**<sup>1</sup> | **R**<sup>2</sup> |
| A | OMe | 4-Iodophenyl |
| B<sup>b</sup> | OMe | 4-Fluorophenyl |
| C | H | 4-Fluorophenyl |
| D | H | 4-Bromophenyl |
| E | H | 2,6-Dichlorophenyl |
| F | H | 2,4-Difluorophenyl |
| G | H | Phenyl |
| H | 4-Tolyl |
| I | OMe | Methyl |
| J | OMe | 4-Chloromethyl |
| K | H | 4-Chloromethyl |
| L | OMe | 4-Chloromethyl |

\textsuperscript{a}See ref. 22 for preparation. \textsuperscript{b}Corresponds with compound 12 from Scheme 2.
60 GF_{254} that were visualised under UV light (at 254 and/or 360 nm). Microwave irradiation experiments were performed in a sealed Pyrex tube using a self-tunable CEM Discover or CEM Explorer focused monomodal microwave synthesizer at the given temperature using the instrument’s in-built IR temperature measuring device, by varying the irradiation power (initial power given in parentheses).

Fully characterized compounds were chromatographically homogeneous. Melting points were determined on a Kofler hot stage apparatus or Stanford Research Systems OptiMeltp and are uncorrected. Specific rotations were measured at the indicated temperature (in °C) using a ADP440 polarimeter (Bellingham + Stanley) at the sodium D line and are given in deg cm$^{-1}$ g$^{-1}$ dm$^{-1}$ with concentration c in $10^{-2}$ g cm$^{-3}$. Infrared spectra were recorded in the range 4000–600 cm$^{-1}$ on a Perkin-Elmer 1600 series FTIR spectrometer using an ATR probe or using KBr disks or as a nujol mull for solid samples and thin films between NaCl plates for liquid samples and are reported in cm$^{-1}$. NMR spectra were recorded using a Varian VNMRS instrument operating at 400 or 500 MHz or a Bruker Avance III 400 MHz for $^1$H spectra and 101 or 126 MHz for $^{13}$C spectra; $J$ values were recorded in Hz and multiplicities were expressed by the usual conventions. Low resolution mass spectra were determined using a Waters Q-TOF Ultima using electrospray positive ionization, A Waters LCT premier XE using atmospheric pressure chemical ionization (APcI), an Agilent 6130 single quadrupole with an APCI/electrospray dual source, a Fisons Instrument VG Autospec using electron impact ionization at 70 eV (low resolution) or a ThermoQuest Finnigan LCQ DUO electrospray, unless otherwise stated. TOFMS refers to time-of-flight mass spectrometry, ES refers to electrospray ionization, CI refers to chemical ionization (ammonia), FTMS refers to Fourier transform mass spectrometry, NSI refers to nano-electrospray ionization and EI refers to electron ionization. A number of high resolution mass spectra were obtained courtesy of the EPSRC Mass Spectrometry Service at University College of Wales, Swansea, UK using the ionization methods specified.

**Synthetic procedures**

3-Methoxybenzoylacetonitrile (6) by Claisen ester condensation using NaH as base (Table 2, entry 5). NaH (60% dispersion in mineral oil; 0.49 g, 12.0 mmol) was added portion-wise to a stirred solution of dry MeCN (0.65 mL, 20.3 mmol) in dry toluene (5 mL) at 0 °C under Ar, and the mixture was warmed to room temperature and stirred for 2 h. The reaction mixture was then cooled to 0 °C before dropwise addition of a solution of methyl 3-methoxybenzoate (9) (1.05 g, 6.3 mmol) in dry toluene (5 mL). Once complete, the solution was allowed to warm to room temperature and stirred at 90 °C for 16 h. After cooling, the reaction mixture was filtered and the filtrate was extracted with water (20 mL). The isolated solids were then dissolved in the aqueous extract, acidified to pH 3–4 using aqueous HCl solution (1 M), extracted with EtOAc (3 × 20 mL), dried (Na$_2$SO$_4$) and evaporated in vacuo. Purification by column chromatography on silica gel (dry load), gradient eluting with hexanes to EtOAc–hexanes (2 : 3), and recrystallization (EtOAc–hexanes) gave the *title compound* (0.36 g, 34%) as a colourless solid, mp 87.3–88.2 °C (EtOAc), lit., 42 mp 87–88 °C.}

**Organic & Biomolecular Chemistry**


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3-Methoxybenzoylacetonitrile (6) by Heck coupling, followed by enol ether hydrolysis (Table 1). 3-Methoxycarbonylnitrole (5) (0.28 g, 3.40 mmol) was added to a stirred solution of 3-iodoanisole (0.20 g, 0.86 mmol), base (1.71 mmol), tetrabutylammonium bromide (27 mg, 86 µmol) and Pd(OAc)$_2$ (19 mg, 86 µmol) in water–MeCN (3 : 1) (3 mL) under Ar and the mixture was heated at 90 °C for 20 h (entries 1 and 2) or irradiated at 130 °C or 140 °C for 1–1.5 h in a pressure-rated glass vial (10 mL) using a CEM Discover microwave synthesizer (entries 3–6) by moderating the initial power (150 W). After cooling, the solution was partitioned between water and EtOAc and the aqueous layer was further extracted with EtOAc (2 × 10 mL). The organic extracts were combined, washed with brine (20 mL), dried (Na$_2$SO$_4$) and evaporated in vacuo. Purification by column chromatography on silica gel (dry load), gradient eluting with hexanes to EtOAc–hexanes (2 : 3), gave the *title compound* as a yellow solid, mp 87.0–87.6 °C.
EtOAc (2 × 20 mL). The organic extracts were combined, washed with brine (20 mL), dried (Na$_2$SO$_4$) and evaporated in vacuo. Purification by column chromatography on silica gel (dry load), gradient eluting with hexanes to EtOAc–hexanes (15:85), gave the title compounds [0.26 g, 68%; Z: E = 1:2] as yellow oils [R$_f$ 0.5 (Z)-7 and 0.3 (E)-7 in EtOAc–light petroleum (1:4)] [Found: ES]: 212.0679. C$_{14}$H$_{14}$NO$_2$ [MNa] requires 212.0682; IR (neat) $\nu_{\text{max}}$ cm$^{-1}$ 3069 (C-H str.), 173.0 (C=O str.), 1596 (N-H bend), 8.06 (1H, d, J = 12, NH), 7.57 (1H, br. s, 2-CH), 7.35 (1H, d, J = 8, 6-CH), 7.10 (1H, d, J = 8, 6-CH), 7.00 (1H, s, 2-CH), 4.24 (3H, s, 3-Ome), 3.84 (3H, s, 3′-Ome); $\delta_H$/ppm [ES]-7 7.40–7.33 (2H, 5′,6′-CH), 7.30 (1H, s, 2′-CH), 7.03 (1H, d, J = 7, 4′-CH), 4.65 (1H, s, 2-Ch), 3.85 (6H, app s, 3′- and 3″-Ome); $\delta_C$/ppm [ES]-7 170.3 (3-C), 159.7 (3′-C), 135.3 (1-C), 129.7 (5′-C), 118.9 (6′-CH), 117.3 (CN), 116.7 (4′-CH), 112.2 (2′-CH), 71.2 (2-Ch), 59.6 (3-Ome), 55.4 (3′-Ome); $\delta_C$/ppm [ES]-7 173.0 (3-C), 159.4 (3′-C), 134.1 (1′-C), 129.5 (5′-CH), 120.3 (6′-C), 118.4 (CN), 117.4 (4′-CH), 112.9 (2′-CH), 69.9 (2-Ch), 56.8 (3′-Ome), 55.4 (3″-Ome); $\text{m/z}$ (El) 189 (M$^+$, 100%), 132 (29), 69 (39).

**Methyl 3-methoxybenzoate (9) using conductive heating.** A catalytic amount of concentrated H$_2$SO$_4$ (0.81 mL, 0.20 mmol) was added to a stirred solution of 3-methoxybenzoic acid (8) (1.48 g, 9.7 mmol) in MeOH (10 mL) and the mixture was stirred at reflux for 3 h. After cooling to room temperature, the solvent was evaporated in vacuo and the residue was partitioned between water and EtO. The ethereal solution was washed with saturated aqueous NaHCO$_3$ solution, dried (Na$_2$SO$_4$) and evaporated in vacuo to give the title compound (1.52 g, 94%) as a colourless oil, with identical physical and spectroscopic properties.

2-(3-Methoxybenzoyl)-3-(phenylacetonitrile (11). A solution of 3-methoxybenzylacetanilide (6) (0.20 g, 1.1 mmol) and N,N′-diphenylformamidine (10) (0.24 g, 1.2 mmol) in dry xylene (7.5 mL) was irradiated at 180 °C under Ar for 20 min in a pressure-rated glass tube (35 mL) using a CEM Discover microwave synthesizer by moderating the initial power (200 W). After cooling in a stream of compressed air, the solution was diluted with hexanes to give a precipitate. Isolation by gravity filtration gave the title compound (0.23 g, 74%) as a colourless solid, mp 107.4–108.8 °C (lit.,$^{22}$ mp 105 °C) [Found: ES]: 279.1126. C$_{17}$H$_{17}$N$_2$O$_2$ [MH] requires 279.1128; IR (neat) $\nu_{\text{max}}$ cm$^{-1}$ 3062 (C-H str.), 2921 (C-H str.), 2204 (C=C str.), 1634 (C=C str.), 1596 (N-H bend), 1571 (C=C str.), 1392 (C=C str.), 1313 (C=C str.), 1225 (C=C str.), 1044 (C-O str.), 990 (C-H bend), 867 (N-H wag); $^1$H NMR (500 MHz, CDCl$_3$) $\delta_H$/ppm 12.76 (1H, d, J = 12, NH), 8.06 (1H, d, J = 12, 3′-CH), 7.57 (1H, d, J = 8, 6′-CH), 7.46 (3H, m, 2′, 3′, and 5″-CH), 7.40 (1H, t, J = 8, 5′-CH), 7.30 (1H, m, 4′-CH), 7.23 (2H, d, J = 8, 2′- and 6′-CH), 7.11 (1H, d, J = 8, 6′-CH), 3.89 (3H, s, Me); $^1$C NMR (126 MHz, CDCl$_3$) $\delta_C$/ppm 192.1 (C), 159.6 (3′-C), 154.1 (3-Ch), 139.2 (1′-C), 138.1 (1′′-C), 130.2 (3′, 5″-C), 129.4 (5′-CH), 126.6 (4′-CH), 120.5 (6′-CH), 120.3 (1-CN), 118.9 (4′-CH), 117.9 (2″, 6′-CH), 112.7 (2′-CH), 83.5 (2-C), 55.5 (Me); $\text{m/z}$ (El) 278 (M$^+$, 49%), 277 (M − H$^+$), 100, 135 (52).

- [Amino-1-(4-fluorophenyl)-1H-pyrazol-4-yl]-3-methoxyphenyl ketone (12). A mixture of 2-(3-methoxybenzoyl)-3-(phenylamino)acrylonitrile (11) (0.13 g, 0.46 mmol), 4-fluorophenylhydrazine hydrochloride (3-HCl) (73 mg, 0.47 mmol) and triethylamine (20 µL, 140 µmol) in ethanol (3 mL) was irradiated at 140 °C for 1 h in a pressure-rated glass vial (10 mL) using a CEM Discover microwave synthesizer by moderating the initial power (150 W). After cooling in a stream of compressed air, the solvent was evaporated in vacuo. Purification by column chromatography on silica gel (dry load), gradient eluting with hexanes to EtOAc–hexanes (3:7), gave the title compound (0.11 g, 86%) as a pink solid, mp 130.1–132.0 °C [Found: ES]: 312.1138. C$_{17}$H$_{17}$F$_3$N$_2$O$_3$ [MH] requires 312.1143; IR (neat) $\nu_{\text{max}}$ cm$^{-1}$ 3318 (N-H str.), 2925 (C-H str.), 1610 (C=C str.), 1595 (N-H bend), 1539 (C=C str.), 1286 (C-N str.), 1223 (C-O str.), 1051 (C-O str.), 838 (N-H wag); $^1$H NMR (1200 MHz, CDCl$_3$) $\delta_H$/ppm 7.81 (1H, s, 3′-CH), 7.56 (2H, m, 2′, 6′-CH), 7.42 (2H, m, 5′, 6′-CH), 7.35 (1H, s, 2′-CH), 7.25 (2H, m, 3′, 5″-CH), 7.11 (1H, m, 4′-CH), 6.04 (2H, bs, excl. D$_2$O, NH$_2$), 3.89 (3H, s, Me); $^1$C NMR (101 MHz, CDCl$_3$) $\delta_C$/ppm 189.4 (C), 162.2 (d, $\delta_{J_{CF}}$ = 250, 4′-CF), 159.8 (3′-C), 150.6 (5-C), 142.0 (3-Ch), 141.1 (1′-C), 133.3 (d, $\delta_{J_{CF}}$ = 4, 1′-C), 129.5...
[5-Amino-1-(4-fluorophenyl)-1H-pyrazol-4-yl]-3-hydroxyphenyl ketone (13). Boron tribromide solution (1 M in CH₂Cl₂; 1.7 mL, 1.70 mmol) was added dropwise to a solution of [5-amino-1-(4-fluorophenyl)-1H-pyrazol-4-yl]-3-methoxyphenyl ketone (12) (0.10 g, 0.34 mmol) in dry CH₂Cl₂ (1 mL) at 0 °C under Ar. The mixture was allowed to warm to room temperature and stirred for 20 h. Water (15 mL) was then added cautiously and the mixture was extracted with EtOAc (3 × 15 mL). The organic extracts were combined, washed with brine, dried (MgSO₄) and evaporated in vacuo to give the title compound (0.10 g, quant.) as a brown solid, mp 192.2–194.9 °C (Found: C 70.94; H 4.76; N 12.11. Calculated: C 71.05; H 4.70; N 12.19%).

IR (neat) νmax/cm⁻¹ 3212 (N–H str.), 1592 (C = O str.), 1053 (C – O str.), 839 (NH); 1HNMR (500 MHz, d₄-DMSO) δH/ppm 7.80 (1H, s, 3-CH), 6.66 (1H, d, J = 7.6, 2″-CH), 3.18 (1H, s, 2CH), 2.03 (3H, s, 2″-CH), 1.75 (1H, s, 3″-CH), 1.21 (3H, t, J = 7.1, 3′-CH). 13C NMR (125 MHz, d₄-DMSO) δCH ppm 187.5 (C), 135.0 (d, JCF = 3, 1′-C), 131.1 (5″-CH), 128.5 (d, JCF = 9, 2″-CH), 120.6 (6″-CH), 120.1 (4″-CH), 118.0 (d, JCF = 24, 3″-CH, 5″-CH), 116.0 (2″-CH), 105.7 (4″-C); m/z [EI] 297 (M⁺, 99%), 296 [M – H]⁺, 204 (31).

5-Amino-1-(4-fluorophenyl)-4-[3-{2(S)-3-dihydroxypropoxy}benzoyl]pyrazole (RO3201195) (1). (S)-2,2-Dimethyl-1,3-dioxolan-4-ylmethyl p-toluenesulphonate (14) (0.60 mL, 2.5 mmol) and anhydrous K₂CO₃ (0.59 g, 4.25 mmol) were added to a solution of [5-amino-1-(4-fluorophenyl)-1H-pyrazol-4-yl]-3-hydroxyphenyl ketone (13) (0.48 g, 1.61 mmol) in dry DMSO (15 mL) under argon and the mixture was heated at 100 °C for 40 h. After cooling, the solution was diluted with water (25 mL) and extracted with EtOAc (3 × 20 mL). The organic extracts were combined, washed with brine, dried (MgSO₄) and evaporated in vacuo. Purification by column chromatography on silica gel (dry load), gradient eluting with light petroleum to EtOAc–light petroleum (2:3), gave [5-amino-1-(4-fluorophenyl)-1H-pyrazol-4-yl]-{3-[{R(2,2-dimethyl-1,3-dioxolan-4-yl)-methoxy]phenyl} ketone (15), which was dissolved in MeOH–water (4:1) (4 mL). p-Toluene sulfonic acid monohydrate (40 mg, 0.21 mmol) was added and the solution was heated at 50 °C for 1 h. After cooling, the solvent was evaporated in vacuo. The residue was dissolved in EtOAc, washed with saturated aqueous NaHCO₃ solution, dried (MgSO₄) and evaporated in vacuo. Purification by column chromatography on silica gel (dry load), gradient eluting with light petroleum to EtOAc, followed by recrystallization (EtOAc–hexanes) gave the title compound (0.22 g, 37%) as a colourless solid, mp 154.1–154.6 °C (EtOAc) (Found: C 73.19; H 4.56; N 12.56. Calculated: C 73.26; H 4.63; N 12.63%).

Cells and cell growth

Human adult dermal primary Werner syndrome AG05229 and AG03141 fibroblasts were obtained from the Coriell Cell Repositories (Camden, NJ, USA). WS²⁺⁵ cells are AG03141 fibroblasts that have been immortalised by the ectopic expression of human enzyme telomerase and have been described previously. Cells were grown in DMEM growth medium as previously described. Cell proliferation rates for the WS²⁺⁵ cells were measured as cumulated population doublings (CPDs) divided by the ratio of phospho-protein/total protein (Fig. 2d and e). In this system activation of p38α by anisomycin activates the kinase MK2 that then phosphorylates the small heat shock protein HSP27. As MK2 is the major HSP27 kinase, the activity of p38α can be assessed by the phosphorylation status of HSP27.

Immunoblot assays for stress kinase activity in WS cells

The ability of SB203580 and RO3201195 to inhibit the p38α signalling pathway was tested in human immortalised HCA2 cells using an ELISA system (obtained from Cell Signalling, NEB, UK) as previously described. Kinase activity was detected using antibodies specific for HSP27 and its phosphorylated form, the degree of activation being measured as the ratio of phospho-protein/total protein (Fig. 2d and e). In this system activation of p38α by anisomycin activates the kinase MK2 that then phosphorylates the small heat shock protein HSP27. As MK2 is the major HSP27 kinase, the activity of p38α can be assessed by the phosphorylation status of HSP27.
Conclusions

This manuscript describes the chemical synthesis and biological evaluation of a p38α MAPK inhibitor, RO3201195 (1), in WS cells. Two complementary routes for the chemical synthesis of a key intermediate, benzoylacetonitrile 6, are described, both of them involving the use of microwave dielectric heating for rapid reaction kinetics. Of these two routes, a classical approach based upon a Claisen ester condensation was found to be the most efficient. The benzoylacetonitrile intermediate 6 was transformed by Knoevenagel condensation, followed by cyclocondensation with a hydrazine, both under microwave irradiation, to give the core pyrazole motif. Phenoxoamide alklylation by S$_2$ displacement of tosylate, followed by ketal deprotection, gave the target inhibitor in a sequence of 7 steps and 15% overall yield.

The effects of RO3201195 (1) on cellular proliferation were first examined using WS$_{3T3}$ cells, where it was found that the inhibitor elicited a very similar response to the prototypical p38 MAPK inhibitor SB203580. The maximal increase seen in the proliferation rate of WS$_{3T3}$ cells was 19% at 10.0 μM concentration of RO3201195 (1). At this concentration, the inhibitor completely inhibited p38α but did not inhibit total JNK activity in WS$_{3T3}$ cells, as determined by immunoblot assay. Following these findings, the cellular senescence of primary Werner syndrome fibroblast strain WAG05229 was investigated on treatment with SB203580 or RO3201195 (1). Despite the fact that these inhibitors showed very different kinase specificities at the concentrations used, the effects on WS cells were essentially identical. Thus, we concluded that the effects on proliferation of treating WS$_{3T3}$ cells or primary WS fibroblasts with SB203580 or RO3201195 (1) resulted from inhibition of p38α. This was further supported by studying the effects of a pyrazolyl ketone library on WS$_{3T3}$ cells at a given concentration, which showed a linear relationship between the proliferation rate and % p38α activity. Thus, we conclude RO3201195 (1) is a useful chemical tool for dissecting signalling pathways in human cells in order to understand the effect of p38 MAPK signalling upon cell proliferation in this human progeroid syndrome.

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Notes and references

4 M. Back and G. K. Hansson, Nat. Rev. Cardiol., 2015, 12, 199–211.