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

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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

P38 $\alpha$  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.<sup>1,2</sup> The reduction of pro-inflammatory cytokine levels offers a means for the treatment of inflammatory disorders such as rheumatoid arthritis and atherosclerosis.<sup>3,4</sup> In addition to inflammatory disorders, p38 $\alpha$  is known to play a role in the resistance of various cancers to chemotherapy, cancer proliferation and metastasis.<sup>5,6</sup> This makes p38 $\alpha$  a compelling therapeutic target for the design of safe and efficacious inhibitors suitable for clinical investigation.<sup>2,7</sup> Following the discovery that pyridinylimidazole p38 $\alpha$  inhibitors such as SB203580 mediate multiple cellular responses,<sup>1</sup> including the production of inflammatory cytokines, a wide variety of structurally-distinct chemotypes<sup>8,9</sup> have been discovered to inhibit this enzyme with notable differences in binding motif.<sup>10</sup> Many of these have been used in stage II or III clinical trials.<sup>11</sup>

Werner syndrome (WS) is a rare autosomal recessive disorder.<sup>12</sup> 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 p38 $\alpha$ .<sup>13-15</sup> 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 p38 $\alpha$  inhibitor SB203580 to WS cells rescues all of the features of accelerated replicative decline.<sup>13</sup> This observation suggested that the abbreviated life span of WS cells is due to stress-induced growth arrest mediated by p38 $\alpha$  MAPK, which we speculate is transduced from the frequently stalled DNA replication forks observed in these cells.<sup>16</sup> It also offers a means to clinically regulate this process and, by therapeutic means, intervene in a premature ageing syndrome.<sup>16</sup>

However, it has been shown that SB203580 is not specific for p38 $\alpha$ , but also inhibits the related N-terminal c-Jun kinases (JNKs) that may play a role in cellular senescence.<sup>17-19</sup> Thus more work is needed using inhibitors that do not target the JNKs to verify the role played by p38 $\alpha$  in WS cells. In addition, SB203580 is not suitable for possible *in vivo* use.<sup>11</sup>

This manuscript describes a rapid route to one such inhibitor, RO3201195 (1),<sup>20</sup> the study of this inhibitor in WS cells, and its comparison with the canonical p38 $\alpha$  inhibitor SB203580 to investigate the role of p38 $\alpha$  MAPK signal transduction in replicative senescence. This pyrazolyl ketone p38 $\alpha$  MAPK inhibitor progressed to clinical trials, is orally bioavailable, exhibits high kinase selectivity, and inhibited the

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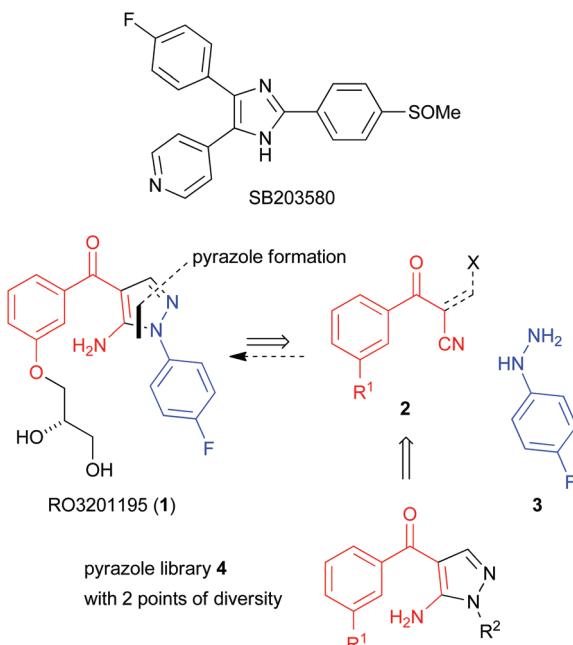
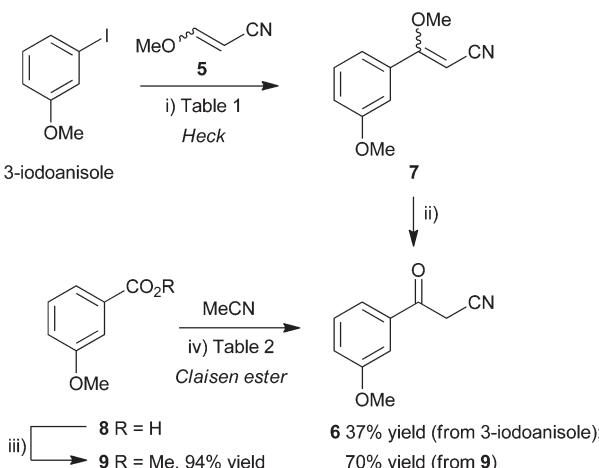


Fig. 1 P38 MAPK inhibitors SB203580 and RO3201195 (1), a pyrazolyl ketone library 4 and disconnective approach to 1.

production of the cytokines IL-6 and TNF $\alpha$  upon lipopolysaccharide (LPS) challenge in rats.<sup>20</sup> 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.<sup>13,21</sup> 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 benzoylnitrile 2, derivatized by homologation prior to heterocycle formation (Fig. 1). This approach had been successful for the synthesis of a pyrazolyl ketone library (4)<sup>22</sup> 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  $\beta$ -ketonitrile derivative 2, as a precursor for pyrazole formation, was first investigated using the Pd-catalyzed Heck reaction<sup>23,24</sup> 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  $\alpha$ - or  $\beta$ -substituted enol ethers are known and have been shown to provide efficient access to 1-arylpropanone derivatives.<sup>25</sup> However, although there are numerous examples of Heck reactions of  $\alpha,\beta$ -disubstituted alkenes such as methylacrylates,<sup>26</sup> 3-ethoxy-



Scheme 1 Synthesis of 3-methoxybenzoylacetonitrile (6) by Heck coupling (top) or Claisen ester condensation (bottom). Reagents and conditions: (i) see Table 1; (ii)  $\text{CHCl}_3\text{--MeOH}$  (1 : 4), microwaves,  $110\text{ }^\circ\text{C}$ , 30 min; (iii)  $\text{MeOH}, \text{H}_2\text{SO}_4$ , reflux, 2.5 h (90%); or  $\text{MeOH}, \text{H}_2\text{SO}_4$ , microwaves,  $110\text{ }^\circ\text{C}$ , 10 min (94%); (iv) see Table 2;  $\text{HCl}$  (aq).

acrylates,<sup>27</sup> cinnamates,<sup>28</sup> crotonates<sup>29</sup> and alkoxypropenes,<sup>30</sup> there are very few examples of  $\beta$ -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.<sup>31</sup> Furthermore,  $\alpha,\beta$ -disubstituted alkenes may suffer from steric constraints and so, although direct, this approach for the synthesis of benzoylacetonitriles was untested. Ortart *et al.*<sup>32</sup> did describe the arylation of an  $\alpha$ -methoxyacrylate back in 1987 using aryl iodides, palladium acetate (3 mol%),  $\text{NaHCO}_3$  base and tetrabutylammonium chloride as additive. Using Jeffery's conditions for Heck-type processes as a starting point,<sup>33</sup> methods related to the conditions for arylation of acrylonitrile with aryl iodides<sup>34</sup> were investigated in the Heck reaction of 3-iodoanisole and 3-methoxyacrylonitrile (5). The use of  $\text{Pd}(\text{OAc})_2$  (10 mol%) as catalyst in  $\text{MeCN--H}_2\text{O}$  (3 : 1) (Table 1) under microwave-assisted heating at  $130\text{ }^\circ\text{C}$  for 1.5 h (entry 4) with  $\text{K}_2\text{CO}_3$  as base and tetrabutylammonium bromide (TBAB) as additive gave, following aqueous work up, the  $\alpha$ -aryl enol ether 7 as a mixture of (*E*)- and (*Z*)-diastereoisomers, in a 2 : 1 ratio, as determined by  $^1\text{H}$  NMR spectroscopic analysis. The diastereoisomers could be separated by column chromatography, albeit in variable and poor yield. Low conversions and poor diastereoselectivity has been observed before in Heck reactions of  $\beta$ -alkoxyacrylonitriles<sup>25</sup> 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 diastereoisomeric mixture 7 required very forcing conditions. Simple treatment of the Heck reaction mixture with acid under ambient conditions failed to provide  $\beta$ -ketonitrile 6. The hydrolysis of related alkoxyacrylonitriles were notable, by their absence,



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

Entry	Base	Additive	Conditions <sup>a</sup>	Yield <sup>b</sup> (%)
1	K <sub>2</sub> CO <sub>3</sub>	TBAB	Conductive heating, 90 °C, 24 h	36
2	NaHCO <sub>3</sub>	TBAB	Conductive heating, 90 °C, 24 h	34
3	K <sub>2</sub> CO <sub>3</sub>	TBAB	Microwaves, <sup>c</sup> 130 °C, 1 h	26
4	K <sub>2</sub> CO <sub>3</sub>	TBAB	Microwaves, <sup>c</sup> 130 °C, 1.5 h	20
5	NaHCO <sub>3</sub>	TBAB	Microwaves, <sup>c</sup> 130 °C, 1.5 h	37
6	NaHCO <sub>3</sub>	TBAB	Microwaves, <sup>c</sup> 140 °C, 1 h	19
7	K <sub>2</sub> CO <sub>3</sub>	DPPP	Microwaves, <sup>c</sup> 150 °C, 1.5 h	31

<sup>a</sup> Isolated yield of benzoylacetonitrile 6. <sup>b</sup> Reaction of 3-iodoanisole (1 equiv.), 3-methoxyacrylonitrile (5) (4 equiv.), Pd(OAc)<sub>2</sub> (10 mol%), base (2 equiv.) and additive (10 mol%) in MeCN–H<sub>2</sub>O (3 : 1) under the given conditions, followed by aqueous work up, microwave irradiation in cHCl–MeOH (1 : 4) at 110 °C (hold time) for 30 min (by moderation of initial power, 150 W) and aqueous work up, followed by purification by column chromatography on silica. <sup>c</sup> Microwave irradiation in a sealed tube at the given temperature, as measured by the instrument's in-built IR sensor, for the given time (hold time) by moderation of the initial power (150 W).

from the study of Doucet and Santelli following Heck reaction of 3-ethoxyacrylonitrile, catalyzed by a tetraphosphane/palladium complex.<sup>25</sup> 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%).

Efforts to improve the overall yield of this process by changing the base from K<sub>2</sub>CO<sub>3</sub> to NaHCO<sub>3</sub>, in accordance with Heck methods for the synthesis of cinnamonnitriles,<sup>34</sup> carrying out the hydrolysis in cHCl–MeOH (1 : 4) under microwave irradiation as before (Table 1, entry 2), gave no improvement in yield (34%). Similarly, carrying out the Heck reaction in a sealed tube under microwave irradiation using K<sub>2</sub>CO<sub>3</sub> at 130 °C for 1 h (entry 3) or 1.5 h (entry 4) was poorly efficient. The microwave-assisted reaction using NaHCO<sub>3</sub> at 130 °C for 1.5 h (entry 5) did give a modest yield of product (37%), similar to the longer conductive heating processes, but this was not improved further by raising the temperature of reaction to 140 °C (entry 6) or to 150 °C in the presence of an alternative additive DPPP (10 mol%) (entry 7). Although the route had been successful for the preparation of ketonitrile 6, the need to isolate enol ether 7, in order to carry out the hydrolysis under high-temperature conditions, and the modest yield for the overall process (entry 5 gave the best yield, at 37%) meant that an alternative route had to be sought.

Considering the original approach to RO3201195 (1) by Roche,<sup>20</sup> efforts were made to access ketonitrile 6 by a more traditional Claisen ester approach. Fischer esterification of 3-methoxybenzoic acid (8) in methanol using sulfuric acid as catalyst at reflux for 3 h gave methyl ester 9 in good yield (90%). This was improved further by carrying out the process in a sealed tube under microwave irradiation at 110 °C for 10 min to give methyl ester 9 in 94% yield (Scheme 1, bottom).

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

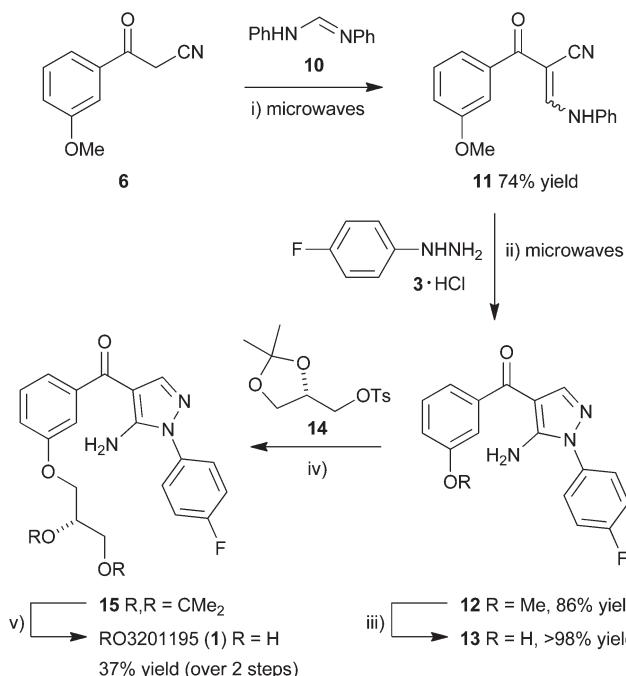
Entry	Base	Conditions	Yield <sup>a</sup> (%)
1	NaOEt	MeCN, RT; reflux, 24 h	9
2	NaOMe	MeCN, RT; reflux, 24 h	9
3	NaH	THF, reflux, 1 h	15
4 <sup>b</sup>	NaH	THF, microwaves, 150 °C, 1 h	35
5	NaH	PhMe, 90 °C, 16 h	34
6	LDA	THF, –78 °C, 1.5 h	18
7	LDA	THF, –50 °C, 3 h	70

<sup>a</sup> Isolated yield of benzoylacetonitrile 6 from methyl benzoate 9 after purification by column chromatography on silica. <sup>b</sup> Microwave irradiation in a sealed tube at the given temperature, as measured by the instrument's in-built IR sensor, for the given time (hold time) by moderation of the initial power.

A well-established method<sup>35</sup> 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,<sup>17,36,37</sup> the aminopyrazole core motif of BIRB 796,<sup>38</sup> and pyrazolyl ketone libraries,<sup>22,39</sup> benzoylacetonitrile 6 was homologated in a microwave-assisted Knoevenagel condensation using *N,N'*-diphenylformamidine (10) in xylenes at 180 °C for 20 min to give enaminone 11 in good yield (74%) (Scheme 2). Cyclocondensation by microwave irradiation with the hydrochloride salt of 4-fluorophenylhydrazine (3) in the presence of Et<sub>3</sub>N in EtOH at 140 °C for 1 h gave 5-aminopyrazole 12 in superior yield (86%) to a range of other methods,<sup>39</sup> after purification by column chromatography. Protodemethylation using BBr<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> (1 M) at RT overnight gave the corresponding phenol 13 in quantitative yield, which was reacted in a S<sub>N</sub>2 displacement reaction with ketal-protected tosyl glycerate 14 under basic conditions. The reaction in DMF in the presence of K<sub>2</sub>CO<sub>3</sub> at 80 °C for 24 h gave only a very low yield of product 15 (9%)<sup>39</sup> but switching to DMSO and carrying



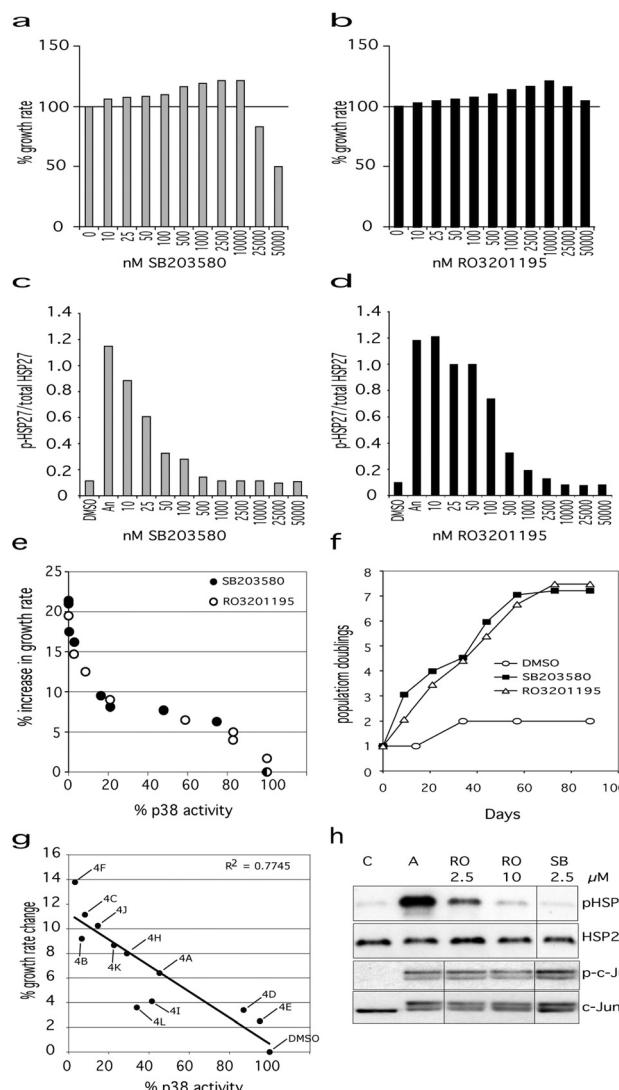


**Scheme 2** Microwave-assisted synthesis of RO3201195. Reagents and conditions: (i) xylenes, microwaves, 180 °C, 20 min; (ii) Et<sub>3</sub>N, EtOH, 140 °C, 1 h; (iii) BBr<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> (1 M), 0 °C-RT, 18 h; (iv) K<sub>2</sub>CO<sub>3</sub>, DMSO, 100 °C, 40 h; (v) TsOH, MeOH-H<sub>2</sub>O, 50 °C, 18 h.

out the S<sub>N</sub>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.<sup>22,39</sup> It was prepared in 15% overall yield in 7 separate steps from commercially-available material, three of them employing microwave irradiation, to provide rapid access to material suitable for examination in WS cells.

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<sup>tert</sup> cells (see materials and methods for a description of these cells). Despite being immortal, these telomeredized cells retain the slow proliferation rate typical of primary WS cells and show a similar response to SB203580 treatment.<sup>14</sup> Thus, these cells can be used to test the effects of modulators of the p38-signaling pathway prior to their use on primary cells.

WS<sup>tert</sup> cells were grown in standard medium supplemented with SB203580 at a final concentration ranging from 10 nM to 50 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<sub>50</sub> for p38α of approximately



**Fig. 2** Effects of p38α inhibitors on Werner syndrome cells. Effects of SB203580 (a) and RO3201195 (b) on the proliferation rate of WS<sup>tert</sup> cells, with 100% representing the DMSO control. Titration curves for inhibition of p38α activity for SB203580 (c) and RO3201195 (d) measured as the ratio of phosphorylated HSP27 to total HSP27 (measured by ELISA). For (a) to (d) DMSO are control cells, 'An' are cells treated with anisomycin to activate p38α, 10 to 50 000 are cells treated with p38 inhibitors at the indicated concentration. (e) Plot of % p38α activity versus % increase in WS<sup>tert</sup> cell proliferation rate for both SB203580 and RO3201195. (f) Proliferation of Werner syndrome AG05229 primary fibroblasts in the presence of SB203580 (2.5 μM) or RO3201195 (10 μM) measured as population doublings versus days. (g) Plot of % increase in proliferation rate of WS<sup>tert</sup> cells against % p38α activity using pyrazolyl ketone library 4. See Table 3 for compound identities. (h) Immunoblot showing that neither SB203580 nor RO3201195 inhibit JNK at the optimal concentrations for their effects on cellular proliferation rates. C = DMSO control, A = cells treated with anisomycin to activate p38α and JNK, and the other three lanes show the effect of the given inhibitor concentrations in anisomycin-treated cells.

30 nM as measured by us, and others.<sup>40,41</sup> SB203580 treatment resulted in an increased proliferation rate of the WS<sup>tert</sup> cells compared to DMSO-treated control cells even at the lowest con-



centration used, with the effect on proliferation rate increasing steadily with increasing SB203580 concentration and reaching a maximum between 2.5  $\mu$ M and 10  $\mu$ M. When the level of p38 $\alpha$  activity was plotted against the effects upon proliferation rate a sigmoid curve was found (Fig. 2e). At 2.5  $\mu$ M 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  $\mu$ M.<sup>39</sup> In this work, RO3201195 inhibited p38 $\alpha$  at a minimum concentration of 25 nM, with the maximal effect seen at 10.0  $\mu$ M (Fig. 2d). RO3201195 had an increasing effect on the proliferation rate of WS<sup>tert</sup> cells up to 10.0  $\mu$ M concentration, thereafter becoming inhibitory (Fig. 2b). As seen for SB203580, when the level of p38 $\alpha$  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<sup>tert</sup> cells was 19% at 10.0  $\mu$ M (Fig. 2b and e).

The above data show that the optimal concentration of these inhibitors for the maximal effects on WS<sup>tert</sup> cells are 2.5  $\mu$ M for SB203580 and 10.0  $\mu$ M for RO3201195. The inhibitors completely inhibited p38 $\alpha$  but did not inhibit total JNK activity in WS<sup>tert</sup> cells at these concentrations as measured by analyzing the phosphorylation of their downstream targets HSP27 and c-Jun by immunoblot (Fig. 2h). WS<sup>tert</sup> cells are stimulated with anisomycin that activates p38 $\alpha$  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  $\mu$ M and SB203580 at 2.5  $\mu$ M 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 p38 $\alpha$  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  $\mu$ M was used, whereas for RO3201195 10.0  $\mu$ M concentration was employed, as these were the lowest concentrations that resulted in maximal proliferation rate and 100% p38 $\alpha$  inhibition (see Fig. 2). The cells were re-fed with DMEM containing the inhibitors as described previously.<sup>15</sup> 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<sup>tert</sup> cells and primary WS fibroblasts result from the inhibition of p38 $\alpha$ .

**Table 3** Pyrazolyl ketone library 4 studied in WS<sup>tert</sup> cells<sup>a</sup>

4	R <sup>1</sup>	R <sup>2</sup>	Chemical Structure
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	
H	H	Phenyl	
I	OMe	4-Tolyl	
J	OMe	Methyl	
K	H	4-Chloromethyl	
L	OMe	4-Chloromethyl	

<sup>a</sup> See ref. 22 for preparation. <sup>b</sup> Corresponds with compound 12 from Scheme 2.

Finally, we used a pyrazolyl ketone library 4 (Table 3) of compounds related to RO3201195<sup>22</sup> for their effects on the proliferation of WS<sup>tert</sup> cells (Fig. 2g). These were used at a concentration of 1.5  $\mu$ M that resulted in a varied degree of p38 $\alpha$  inhibition from approximately 5% to greater than 95% (Fig. 2g and ref. 22). When the effect on the proliferation rate of WS<sup>tert</sup> cells by this pyrazolyl ketone library were plotted against the % p38 $\alpha$  activity (as assessed by ELISA<sup>22</sup>), a linear response was seen (Fig. 2g), providing further support that these effects on cellular proliferation are due to p38 $\alpha$  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<sup>13</sup> is *via* p38 $\alpha$  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.<sup>16</sup> 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.<sup>20</sup> In addition, it has shown therapeutic efficacy *in vivo* in rats in regulating cytokine production upon LPS challenge,<sup>20</sup> 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 Matrix silica 60. Analytical thin layer chromatography was carried out using aluminium-backed plates coated with Merck Kieselgel



60 GF<sub>254</sub> 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 Optimelt 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<sup>3</sup> g<sup>-1</sup> dm<sup>-1</sup> with concentration *c* in 10<sup>-2</sup> g cm<sup>-3</sup>. Infrared spectra were recorded in the range 4000–600 cm<sup>-1</sup> 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<sup>-1</sup>. NMR spectra were recorded using a Varian VNMRS instrument operating at 400 or 500 MHz or a Bruker Avance III 400 MHz for <sup>1</sup>H spectra and 101 or 126 MHz for <sup>13</sup>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 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-Methoxybenzoylacetetonitrile (6) by Claisen ester condensation using LDA as base (Table 2, entry 6).** *n*-BuLi (2.5 M in hexanes; 5.3 mL, 13.2 mmol) was added dropwise to a stirred solution of DIPA (1.9 mL, 13.6 mmol) in dry THF (2 mL) at 0 °C under Ar, and the mixture was stirred at 0 °C for 10 min. In a separate flask, a solution of methyl 3-methoxybenzoate (9) (1.1 g, 6.6 mmol) and dry acetonitrile (0.5 mL, 9.5 mmol) in dry THF (5 mL) was cooled to –50 °C. The cold LDA solution was added dropwise and the reaction mixture was stirred at –50 °C for 3 h. Saturated aqueous NH<sub>4</sub>Cl solution (10 mL) was then added. The solution was allowed to warm to room temperature, extracted with EtOAc (3 × 20 mL), washed with aqueous HCl solution (1 M; 20 mL) and brine (20 mL), dried (MgSO<sub>4</sub>) 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*

(0.82 g, 70%) as a yellow solid, mp 86.3–87.4 °C (MeOH) (lit.<sup>42</sup> mp 87–88 °C) (Found [ES]: 198.0523. C<sub>10</sub>H<sub>9</sub>NO<sub>2</sub>Na [MNa] requires 198.0525); IR (neat)  $\nu_{\text{max}}$ /cm<sup>-1</sup> 2948 (CH str.), 2252 (C≡N str.), 1694 (C=O str.), 1580 (CC), 1451 (CH), 1258 (C–O), 1012 (C–O); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$ <sub>H</sub>/ppm 7.49–7.41 (3H, 2-, 5- and 6-CH), 7.21 (1H, d, *J* = 8, 4-CH), 4.07 (2H, s, CH<sub>2</sub>), 3.88 (3H, s, Me); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>)  $\delta$ <sub>C</sub>/ppm 186.9 (C), 160.2 (3-C), 135.7 (1-C), 130.1 (5-CH), 121.2 (4-CH), 120.9 (6-CH), 113.6 (CN), 112.8 (2-CH), 55.6 (Me), 29.4 (CH<sub>2</sub>); *m/z* (EI) 175 (M<sup>+</sup>, 36%), 135 ([M – CH<sub>2</sub>CN]<sup>+</sup>, 100).

**3-Methoxybenzoylacetetonitrile (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 portionwise 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<sub>2</sub>SO<sub>4</sub>) 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.<sup>42</sup> mp 87–88 °C), with identical spectroscopic properties.

**3-Methoxybenzoylacetetonitrile (6) by Heck coupling, followed by enol ether hydrolysis (Table 1).** 3-Methoxyacrylonitrile (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)<sub>2</sub> (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<sub>2</sub>SO<sub>4</sub>) and evaporated *in vacuo*. The crude residue was dissolved in MeOH (2 mL) in air and concentrated HCl was added (10.2 M; 0.5 mL). The mixture was irradiated at 110 °C for 1 h in a pressure-rated glass vial (10 mL) using a CEM Discover microwave synthesiser by moderating the initial power (150 W). After cooling in a flow of compressed air, 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 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





(MeOH) (lit.,<sup>42</sup> mp 87–88 °C), with identical spectroscopic properties.

**(E)- and (Z)-3-Methoxy-3-(3-methoxyphenyl)prop-2-enenitrile (7) by Heck coupling.** 3-Methoxyacrylonitrile (5) (0.72 g, 8.7 mmol) was added to a stirred solution of potassium carbonate (0.63 g, 4.5 mmol), tetrabutylammonium bromide (80 mg, 0.24 mmol), 3-iodoanisole (0.47 g, 2.0 mmol) and palladium acetate (50 mg, 0.20 mmol) in water–MeCN (4 : 1) (12 mL) under Ar and the solution was stirred at 90 °C for 24 h. After cooling, the solution was partitioned between water and EtOAc and the aqueous layer was further extracted with EtOAc (2 × 20 mL). The organic extracts were combined, washed with brine (20 mL), dried (Na<sub>2</sub>SO<sub>4</sub>) 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*<sub>f</sub> 0.5 (*Z*)-7 and 0.3 (*E*)-7 in EtOAc–light petroleum (1 : 4)] (Found [ES]: 212.0679. C<sub>11</sub>H<sub>11</sub>NO<sub>2</sub>Na [MNa] requires 212.0682); IR (neat)  $\nu_{\text{max}}$ /cm<sup>−1</sup> 3069 (C–H str.), 2950 (C–H str.), 2198 (C≡N str.), 1605 (C=C str.), 1579 (C–C str.), 1437 (C–H bend), 1215 (C–O str.), 1117 (C–O str.), 1047 (C–O str.); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta_{\text{H}}$ /ppm [(*Z*)-7] 7.31 (1H, t, *J* = 8, 5'-CH), 7.11 (1H, d, *J* = 8, 6'-CH), 7.06 (1H, s, 2'-CH), 7.00 (1H, d, *J* = 8, 3'-CH), 4.96 (1H, s, 2-CH), 4.24 (3H, s, 3'-OMe), 3.84 (3H, s, 3'-OMe);  $\delta_{\text{H}}$ /ppm [(*E*)-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); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>)  $\delta_{\text{C}}$ /ppm [(*Z*)-7] 170.9 (3-C), 159.7 (3'-C), 135.3 (1'-C), 129.7 (5'-CH), 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);  $\delta_{\text{C}}$ /ppm [(*E*)-7] 173.0 (3-C), 159.4 (3'-C), 134.1 (1'-C), 129.5 (5'-CH), 120.3 (6'-CH), 118.4 (CN), 117.4 (4'-CH), 112.9 (2'-CH), 69.9 (2-CH), 56.8 (3-OMe), 55.4 (3'-OMe); *m/z* (EI) 189 (M<sup>+</sup>, 100%), 132 (29), 69 (39).

**Methyl 3-methoxybenzoate (9) using conductive heating.** A catalytic amount of concentrated H<sub>2</sub>SO<sub>4</sub> (0.01 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 Et<sub>2</sub>O. The ethereal solution was washed with saturated aqueous NaHCO<sub>3</sub> solution, dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated *in vacuo* to give the *title compound* (1.45 g, 90%) as a colourless oil (Found [ES]: 189.0520. C<sub>9</sub>H<sub>10</sub>O<sub>3</sub>Na [MNa] requires 189.0522); IR (neat)  $\nu_{\text{max}}$ /cm<sup>−1</sup> 2953 (C–H str.), 1718 (C=O str.), 1601 (CC str.), 1276 (C–O str.), 1221 (C–O str.); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta_{\text{H}}$ /ppm 7.64 (1H, d, *J* = 8, 6-CH), 7.57 (1H, br. s, 2-CH), 7.35 (1H, t, *J* = 8, 5-CH), 7.11 (1H, dd, *J* = 8, 2, 4-CH), 3.92 (3H, s, CO<sub>2</sub>Me), 3.86 (3H, s, OMe); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>)  $\delta_{\text{C}}$ /ppm 166.9 (C), 159.6 (3-C), 131.5 (1-C), 129.3 (5-CH), 122.0 (6-CH), 199.5 (4-CH), 144.0 (2-CH), 55.4 (OMe), 52.1 (CO<sub>2</sub>Me); *m/z* (EI): 166 (M<sup>+</sup>, 14%), 135 ([M – OMe]<sup>+</sup>, 39), 93 (100).

**Methyl 3-methoxybenzoate (9) using microwave irradiation.** A catalytic amount of concentrated H<sub>2</sub>SO<sub>4</sub> (0.01 mL, 0.20 mmol) was added to a stirred solution of 3-methoxy-

benzoic acid (8) (1.48 g, 9.7 mmol) in MeOH (10 mL) and the mixture was irradiated at 110 °C for 10 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 flow of compressed air, the solvent was evaporated *in vacuo* and the residue was partitioned between water and Et<sub>2</sub>O. The ethereal solution was washed with saturated aqueous NaHCO<sub>3</sub> solution, dried (Na<sub>2</sub>SO<sub>4</sub>) 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-(phenylamino)acrylonitrile (11).** A solution of 3-methoxybenzoylacetonitrile (6) (0.20 g, 1.1 mmol) and *N,N'*-diphenylformamidine (10) (0.24 g, 1.2 mmol) in dry xylenes (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.,<sup>22</sup> mp 105 °C) (Found [ES]: 279.1126. C<sub>17</sub>H<sub>15</sub>N<sub>2</sub>O<sub>2</sub> [MH] requires 279.1128); IR (neat)  $\nu_{\text{max}}$ /cm<sup>−1</sup> 3062 (C–H str.), 2921 (C–H str.), 2204 (C≡N str.), 1634 (C=O str.), 1596 (N–H bend), 1571 (C–C str.), 1392 (C–C str.), 1313 (C–N str.), 1225 (C–O str.), 1044 (C–O str.), 990 (C–H bend), 867 (N–H wag); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta_{\text{H}}$ /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); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>)  $\delta_{\text{C}}$ /ppm 192.1 (C), 159.6 (3'-C), 154.1 (3-CH), 139.2 (1'-C), 138.1 (1"-C), 130.2 (3", 5"-CH), 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); *m/z* (EI): 278 (M<sup>+</sup>, 49%), 277 ([M – H]<sup>+</sup>, 100), 135 (52).

**[5-Amino-1-(4-fluorophenyl)-1*H*-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  $\mu$ L, 140  $\mu$ mol) 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<sub>17</sub>H<sub>15</sub>FN<sub>3</sub>O<sub>2</sub> [MH] requires 312.1143); IR (neat)  $\nu_{\text{max}}$ /cm<sup>−1</sup> 3318 (N–H str.), 2925 (C–H str.), 1610 (C=O 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); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta_{\text{H}}$ /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, exch. D<sub>2</sub>O, NH<sub>2</sub>), 3.89 (3H, s, Me); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta_{\text{C}}$ /ppm 189.4 (C), 162.2 (d,  $^1J_{\text{CF}}$  = 250, 4'-CF), 159.8 (3"-C), 150.6 (5-C), 142.0 (3-CH), 141.1 (1"-C), 133.3 (d,  $^4J_{\text{CF}}$  = 4, 1'-C), 129.5

(5"-CH), 126.1 (d,  $^3J_{CF} = 9$ , 2', 6'-CH), 120.7 (6"-CH), 117.8 (4"-CH), 116.9 (d,  $^2J_{CF} = 23$ , 3', 5'-CH), 113.0 (2"-CH), 104.8 (4-C), 55.4 (OMe);  $m/z$  (APCI) 312 ( $M^+$ , 100%).

**[5-Amino-1-(4-fluorophenyl)-1*H*-pyrazol-4-yl]-3-hydroxyphenyl ketone (13).** Boron tribromide solution (1 M in  $CH_2Cl_2$ ; 1.7 mL, 1.70 mmol) was added dropwise to a solution of [5-amino-1-(4-fluorophenyl)-1*H*-pyrazol-4-yl]-3-methoxyphenyl ketone (12) (0.10 g, 0.34 mmol) in dry  $CH_2Cl_2$  (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 ( $MgSO_4$ ) and evaporated *in vacuo* to give the *title compound* (0.10 g, quant.) as a brown solid, mp 192.2–194.9 °C (Found [ES]: 298.0984.  $C_{16}H_{13}FN_3O_2$  [MH] requires 298.0986); IR (neat  $\nu_{max}/cm^{-1}$  3320 (N–H str.), 3204 (br. O–H), 3072 (C–H str.), 1610 (C=O str.), 1592 (N–H bend), 1538 (C–C str.), 1310 (C–N str.), 1126 (C–O str.), 841 (N–H wag);  $^1H$  NMR (500 MHz,  $CD_3OD$ )  $\delta_{H}/ppm$  7.78 (1H, s, 3-CH), 7.59 (2H, m, 2', 6'-CH), 7.37–7.29 (3H, 3', 5'- and 5"-CH), 7.23 (1H, d,  $J = 7$ , 6"-CH), 7.18 (1H, s, 2"-CH), 7.00 (1H, dd,  $J = 7$ , 1, 4"-CH);  $^{13}C$  NMR (100 MHz,  $CD_3OD$ )  $\delta_{C}/ppm$  191.6 (C), 164.1 (d,  $^1J_{CF} = 247$ , 4'-CF), 159.2 (3"-C), 153.4 (5-C), 143.6 (3-CH), 142.7 (1"-C), 135.0 (d,  $^4J_{CF} = 3$ , 1'-C), 131.1 (5"-CH), 128.5 (d,  $^3J_{CF} = 9$ , 2', 6'-CH), 120.6 (6"-CH), 120.1 (4"-CH), 118.0 (d,  $^3J_{CF} = 24$ , 3', 5'-CH), 116.0 (2"-CH), 105.7 (4-C);  $m/z$  (EI) 297 ( $M^+$ , 91%), 296 ( $[M - H]^+$ , 100), 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*-toluenesulfonate (14) (0.60 mL, 2.5 mmol) and anhydrous  $K_2CO_3$  (0.59 g, 4.25 mmol) were added to a solution of [5-amino-1-(4-fluorophenyl)-1*H*-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 ( $MgSO_4$ ) 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)-1*H*-pyrazol-4-yl]-{3-[*R*]-2,2-dimethyl-1,3-dioxolan-4-yl}-methoxyphenyl 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 18 h. After cooling, the solvent was evaporated *in vacuo*. The residue was dissolved in EtOAc, washed with saturated aqueous  $NaHCO_3$  solution, dried ( $MgSO_4$ ) 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 [ES]: 372.1353.  $C_{19}H_{19}FN_3O_4$  [MH] requires 372.1354);  $[\alpha]_D^{21} -28$  (c 0.2, EtOAc);  $[\alpha]_D^{22} + 6.5$  (c 0.6, MeOH); IR (neat)  $\nu_{max}/cm^{-1}$  3440 (N–H str.), 3330 (O–H str.), 3229 (O–H str.), 2896 (C–H str.), 1633 (C=O str.), 1597 (NH<sub>2</sub> str.), 1540 (C=N str.), 1498 (C–C str.), 1292 (C–O str.),

1222 (C–F str.), 1053 (C–O str.), 839 (NH<sub>2</sub> wag.);  $^1H$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta_{H}/ppm$  7.80 (1H, s, 3H), 7.61 (2H, m, 2', 6'-CH), 7.47–7.37 (3H, 3', 4'- and 5"-CH), 7.33 (1H, d,  $J = 7$ , 6"-CH), 7.25 (1H, m, 2"-CH), 7.14 (3H, 4"-CH and NH<sub>2</sub>), 4.95 (1H, bs, CHOH), 4.65 (1H, bs,  $CH_2OH$ ), 4.09 (1H, m, OCHH), 3.95 (1H, m, OCHH), 3.82 (1H, m, CHOH), 3.47 (2H, d,  $J = 5$ ,  $CH_2OH$ );  $^{13}C$  NMR (126 MHz,  $d_6$ -DMSO)  $\delta_{C}/ppm$  187.5 (C), 161.1 (d,  $^1J_{CF} = 244$ , 4'-C), 158.7 (3"-C), 151.2 (5-C), 141.4 (3-CH), 140.9 (1"-C), 133.7 (d,  $^4J_{CF} = 3$ , 1'-C), 129.6 (5"-CH), 126.4 (d,  $^3J_{CF} = 9$ , 2', 6'-CH), 120.1 (6"-CH), 117.8 (4"-CH), 116.3 (d,  $^2J_{CF} = 23$ , 3', 5'-CH), 113.3 (2"-CH), 103.5 (4-C), 69.9 (CHOH), 69.8 (CH<sub>2</sub>O), 62.6 (CH<sub>2</sub>OH);  $m/z$  (EI) 371 ( $M^+$ , 10%), 325 (33), 160 (100).

## Cells and cell growth

Human adult dermal primary Werner syndrome AG05229 and AG03141 fibroblasts were obtained from the Coriell Cell Repositories (Camden, NJ, USA). WS<sup>tert</sup> cells are AG03141 fibroblasts that have been immortalised by the ectopic expression of human enzyme telomerase and have been described previously.<sup>14</sup>

Cells were grown in DMEM growth medium as previously described.<sup>15</sup> Cell proliferation rates for the WS<sup>tert</sup> cells were measured as cumulated population doublings (CPDs) divided by the number of days of the experiment concerned and expressed as a percentage of the proliferation rate of control cells (with the control being 100%). The number of CPDs achieved at each passage of the cells was calculated according to the formula: PDs =  $\log(N_t/N_0)/\log 2$ , where  $N_t$  is number of cells counted and  $N_0$  is number of cells seeded. For primary cells the CPDs were plotted *versus* days in culture. For assessing the effects of the various kinase inhibitors the culture medium was supplemented with the inhibitor dissolved in DMSO, with the medium being replaced daily. For controls an equivalent volume of the inhibitor solvent (DMSO) was added to the medium.

## P38 inhibitor assay

The ability of SB203580 and RO3201195 to inhibit the p38 $\alpha$  signalling pathway was tested in human immortalised HCA2 cells using an ELISA system (obtained from Cell Signalling, NEB, UK) as previously described.<sup>39</sup> 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 p38 $\alpha$  by anisomycin activates the kinase MK2 that then phosphorylates the small heat shock protein HSP27. As MK2 is the major HSP27 kinase,<sup>43</sup> the activity of p38 $\alpha$  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 p38 $\alpha$  and JNK signalling pathways in WS<sup>tert</sup> cells was tested by immunoblot detection of the phosphorylated versions of their



downstream targets HSP27 (pHSP27) and c-Jun (p-c-Jun) as previously described.<sup>15,44</sup>

## Conclusions

This manuscript describes the chemical synthesis and biological evaluation of a p38 $\alpha$  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. Phenoxide alkylation by S<sub>N</sub>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<sup>tert</sup> 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<sup>tert</sup> cells was 19% at 10.0  $\mu$ M concentration of RO3201195 (1). At this concentration, the inhibitor completely inhibited p38 $\alpha$  but did not inhibit total JNK activity in WS<sup>tert</sup> 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 specificity profiles at the concentrations used, the effects on WS cells were essentially identical. Thus, we concluded that the effects on proliferation of treating WS<sup>tert</sup> cells or primary WS fibroblasts with SB203580 or RO3201195 (1) resulted from inhibition of p38 $\alpha$ . This was further supported by studying the effects of a pyrazolyl ketone library on WS<sup>tert</sup> cells at a given concentration, which showed a linear relationship between the proliferation rate and % p38 $\alpha$  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

- J. C. Lee, J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, *et al.*, *Nature*, 1994, **372**, 739.
- G. L. Schieven, *Curr. Top. Med. Chem.*, 2005, **5**, 921.
- E. Salgado, J. R. Maneiro, L. Carmona and J. J. Gomez-Reino, *Ann. Rheum. Dis.*, 2014, **73**, 871.
- M. Back and G. K. Hansson, *Nat. Rev. Cardiol.*, 2015, **12**, 199–211.
- V. Grossi, A. Peserico, T. Tezil and C. Simone, *World J. Gastroenterol.*, 2014, **20**, 9744.
- K. Yumoto, M. R. Eber, J. E. Berry, R. S. Taichman and Y. Shiozawa, *Clin. Cancer Res.*, 2014, **20**, 3384.
- C. Dominguez, D. A. Powers and N. Tamayo, *Curr. Opin. Drug Discovery Dev.*, 2005, **8**, 421.
- D. J. Diller, T. H. Lin and A. Metzger, *Curr. Top. Med. Chem.*, 2005, **5**, 953.
- D. M. Goldstein and T. Gabriel, *Curr. Top. Med. Chem.*, 2005, **5**, 1017.
- S. T. Wroblewski and A. M. Doweyko, *Curr. Top. Med. Chem.*, 2005, **5**, 1005.
- J. Saklatvala, *Curr. Opin. Pharmacol.*, 2004, **4**, 372.
- D. Kipling, T. Davis, E. L. Ostler and R. G. Faragher, *Science*, 2004, **305**, 1426.
- T. Davis, D. M. Baird, M. F. Haughton, C. J. Jones and D. Kipling, *J. Gerontol., Ser. A*, 2005, **60**, 1386.
- T. Davis, M. F. Haughton, C. J. Jones and D. Kipling, *Ann. N. Y. Acad. Sci.*, 2006, **1067**, 243.
- T. Davis and D. Kipling, *Biogerontology*, 2009, **10**, 253.
- T. Davis and D. Kipling, *Rejuvenation Res.*, 2006, **9**, 402.
- M. C. Bagley, T. Davis, M. J. Rokicki, C. S. Widdowson and D. Kipling, *Future Med. Chem.*, 2010, **2**, 193.
- J. Bain, L. Plater, M. Elliott, N. Shapiro, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alessi and P. Cohen, *Biochem. J.*, 2007, **408**, 297.
- K. Godl and H. Daub, *Cell Cycle*, 2004, **3**, 393.
- D. M. Goldstein, T. Alfredson, J. Bertrand, M. F. Browner, K. Clifford, S. A. Dalrymple, J. Dunn, J. Freire-Moar, S. Harris, S. S. Labadie, J. La Fargue, J. M. Lapierre, S. Larrabee, F. Li, E. Papp, D. McWeeney, C. Ramesha, R. Roberts, D. Rotstein, B. San Pablo, E. B. Sjogren, O. Y. So, F. X. Talamas, W. Tao, A. Trejo, A. Villasenor, M. Welch, T. Welch, P. Weller, P. E. Whiteley, K. Young and S. Zipfel, *J. Med. Chem.*, 2006, **49**, 1562.
- M. C. Bagley, T. Davis, P. G. S. Murziani, C. S. Widdowson and D. Kipling, *Pharmaceuticals*, 2010, **3**, 1842.
- M. C. Bagley, T. Davis, M. C. Dix, P. G. Murziani, M. J. Rokicki and D. Kipling, *Future Med. Chem.*, 2010, **2**, 203.
- T. Mizoroki, K. Mori and A. Ozaki, *Bull. Chem. Soc. Jpn.*, 1971, **44**, 581.



24 R. F. Heck and J. P. Nolley, *J. Org. Chem.*, 1972, **37**, 2320.

25 A. Battace, M. Feuerstein, M. Lemhadri, T. Zair, H. Doucet and M. Santelli, *Eur. J. Org. Chem.*, 2007, 3122.

26 M. Beller and T. H. Riermeier, *Tetrahedron Lett.*, 1996, **37**, 6535.

27 T. Sakamoto, Y. Kondo, Y. Kashiwagi and H. Yamanaka, *Heterocycles*, 1988, **27**, 257.

28 N. A. Cortese, C. B. Ziegler, B. J. Hrnjez and R. F. Heck, *J. Org. Chem.*, 1978, **43**, 2952.

29 A. F. Littke and G. C. Fu, *J. Am. Chem. Soc.*, 2001, **123**, 6989.

30 C. M. Andersson, A. Hallberg and G. D. Daves, *J. Org. Chem.*, 1987, **52**, 3529.

31 T. Jeffery, *J. Chem. Soc., Chem. Commun.*, 1984, 1287.

32 S. Cacchi, P. G. Ciattini, E. Morera and G. Ortari, *Tetrahedron Lett.*, 1987, **28**, 3039.

33 T. Jeffery, *Tetrahedron*, 1996, **52**, 10113.

34 H. Zhao, M.-Z. Cai and C.-Y. Peng, *Synth. Commun.*, 2002, **32**, 3419.

35 J. B. Dorsch and S. M. McElvain, *J. Am. Chem. Soc.*, 1932, **54**, 2960.

36 M. C. Bagley, M. C. Lubinu and C. Mason, *Synlett*, 2007, 704.

37 M. C. Bagley, M. Baashen, V. L. Paddock, D. Kipling and T. Davis, *Tetrahedron*, 2013, **68**, 8429.

38 M. C. Bagley, T. Davis, M. C. Dix, C. S. Widdowson and D. Kipling, *Org. Biomol. Chem.*, 2006, **4**, 4158.

39 M. C. Bagley, T. Davis, M. C. Dix, P. G. Murziani, M. J. Rokicki and D. Kipling, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 3745.

40 S. P. Davies, H. Reddy, M. Caivano and P. Cohen, *Biochem. J.*, 2000, **351**, 95.

41 K. Godl, J. Wissing, A. Kurtenbach, P. Habenberger, S. Blencke, H. Gutbrod, K. Salassis, M. Stein-Gerlach, A. Missio, M. Cotten and H. Daub, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 15434.

42 C. Ota, S. Kumata and S. Kawaguchi, *EP 1544202A1*, 2005, 56–57.

43 J. X. Shi, X. Su, J. Xu, W. Y. Zhang and Y. Shi, *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, 2012, **302**, L793.

44 T. Davis, M. C. Dix, M. J. Rokicki, A. J. C. Brook, C. S. Widdowson, D. Kipling and M. C. Bagley, *Chem. Cent. J.*, 2011, **5**, 83.

