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Microwave-assisted synthesis of 5-aminopyrazol-4-yl ketones and the p38^{MAPK} inhibitor RO3201195 for study in Werner syndrome cells

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ABSTRACT

5-Aminopyrazol-4-yl ketones are prepared rapidly and efficiently using microwave dielectric heating from β-ketonitriles by treatment with *N*,*N*′-diphenylformamidine followed by heterocyclocondensation by irradiation with a hydrazine. The inhibitory activity of RO3201195 prepared by this methodology was confirmed in hTERT-immortalized HCA2 and WS dermal fibroblasts at 200 nM concentration, both by ELISA and immunoblot assay, and displays excellent kinase selectivity for p38α MAPK over the related stress-activated kinase JNK.

Werner syndrome (WS) is a rare genetic disorder that is used as a model disease to investigate normal human ageing and results from mutation in the *WRN* gene.[1] WS individuals show premature onset of many clinical features of old age, early susceptibility to several major age-related diseases and a shortened median life expectancy (47 years) as a consequence of malignancy and myocardial infarction. This accelerated in vivo ageing is associated with premature senescence of WS cells in vitro that may underlie several features of the rapid ageing seen in WS.[2,3] Normal human cells are capable of only a finite number of divisions in culture after which is triggered a non-proliferative state known as senescence. Whether cellular senescence plays a role in organismal ageing has been hotly disputed,[4] however, senescent cells are known to accumulate during ageing, for example, in the skin,[5] and evidence is growing that supports the hypothesis that this finite proliferative capacity may be a causal agent of normal ageing processes. Senescent cells display deleterious biochemical features, such as a pro-inflammatory phenotype, suggesting a link between replicative senescence and tissue degeneration.[6] Normal and WS fibroblasts use telomere erosion as a cell division 'counter',[7] but recently it has been shown that this telomere-driven senescence synergizes with an additional telomere-independent mechanism in WS.[8] Unlike normal human fibroblasts, proliferating WS cells contain high levels of phosphorylated p38α,[8] a stress-activated MAPK, suggesting that WS cells undergo stress-induced premature senescence.[8,9] If accelerated ageing in WS is related to p38 activation and to accelerated cell ageing, WS may provide a powerful model to link cellular signalling events to the ageing of mitotic tissues in vivo. In addition, blocking the p38α response through the use of a chemical inhibitor has the potential to interfere with the pathology of WS, and so may provide opportunities for therapeutic intervention.

In our previous studies, we tested this hypothesis to dramatic effect in hTERT-immortalized WS dermal fibroblasts and in primary WS cells. When these cells were treated with the prototypical p38 inhibitor SB203580, all of the features of accelerated replicative decline were rescued, including growth rate and cell morphology,[8] indicating that the abbreviated life span of WS cells could be due to a stress-induced growth arrest mediated by p38α MAPK.[9] Subsequently we showed that alternative p38 inhibitors with improved kinase selectivity, including BIRB 796[10] and VX-745,[11] could be prepared using microwave-assisted methods and reported their activity in WS cells. However BIRB 796 also inhibits the related stress-activated JNK kinases,[12] as does SB203580, and as young WS cells show elevated levels of phosphorylated JNK1α1 and JNK2α1 isoforms of JNK (our unpublished data), facile access to another chemotype without JNK inhibitory activity that is orally bioavailable is required to corroborate our findings and thence progress towards a clinical study. The 5-aminopyrazol-4-yl ketone p38 inhibitor RO3201195 (Fig. 1)[13] fits this profile, combining good cellular activity, pharmacokinetic properties, metabolic stability

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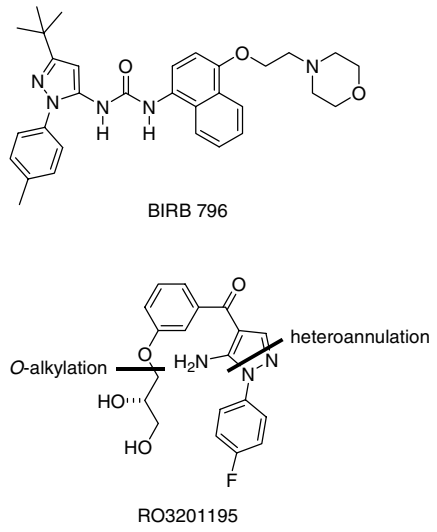
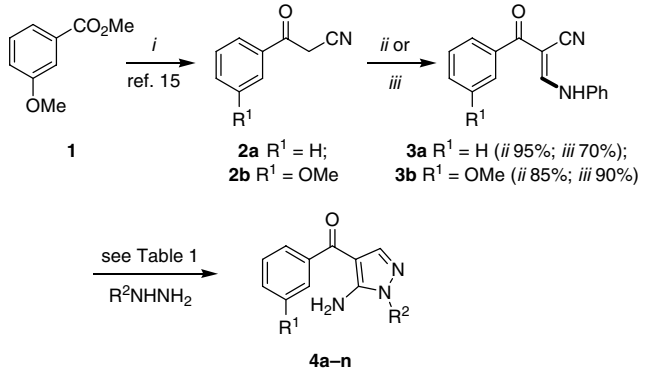


Figure 1. The p38 MAPK inhibitors BIRB 796 and RO3201195, the latter showing our disconnective strategy.

and bioavailability with an excellent selectivity profile as determined in a panel of 105 kinases.

Our synthetic approach to RO3201195 (Fig. 1) utilized a linear strategy: the 5-amino-4-benzoylpyrazole motif would be assembled by regiospecific heterocyclocondensation of the corresponding hydrazine and a β-anilino-α-benzoylacrylonitrile formed by Knoevenagel-type condensation. *O*-Alkylation installs the propyloxy side chain to improve the pharmacokinetic properties as described by Roche.[13] We have shown that related heterocyclocondensation reactions are accelerated dramatically by microwave dielectric heating and used this in the synthesis of polysubstituted pyrazoles[14] and the 5-aminopyrazole core of BIRB 796.[10] For the synthesis of RO3201195, two benzoylacetonitriles, **2a** and **2b**, the latter readily available from **1** by reaction with acetonitrile under basic conditions,[15] were reacted with *N*,*N*′-diphenylformamidine to give cyclocondensation precursors **3a,b** (Scheme 1). Although the Knoevenagel condensation proceeded in good yield for benzoylacetonitriles **2a** and **2b** under traditional conductive heating, microwave irradiation at 180 °C afforded **3b** more efficiently after only 20 min. A variety of conditions were investigated for heterocyclocondensation with a range of hydrazines, present as the free base or hydrochloride salt, including a traditional reflux under conductive heating and microwave irradiation in a sealed vessel at 100–140 °C in the presence or absence of Et_3N (Table 1).[16]



1 **2a** R^1 = H; **2b** R^1 = OMe **3a** R^1 = H (*ii* 95%; *iii* 70%); **3b** R^1 = OMe (*ii* 85%; *iii* 90%) **4a–n**

Scheme 1. Synthesis of pyrazol-4-yl ketones **4a–n**. Reagents and conditions: (i) MeCN, NaOEt, 80 °C (Ref. 15); (ii) PhN=CHNHPh, dry xylenes, reflux, 1.5–2 h; (iii) PhN=CHNHPh, xylenes, microwaves, 180 °C (initial power: **3a**, 100 W; **3b**, 160 W), 20 min.

It was apparent from the microwave irradiation experiments that pyrazolyl ketone formation was, for the most part, rapid and high-yielding. For hydrazine hydrochlorides, the addition of Et_3N to the reaction mixture improved the efficiency significantly. From pyrazolyl ketone **4b**, further elaboration to RO3201195 involved deprotection of the phenolic ether by boron tribromide-mediated protodemethylation, followed by *O*-alkylation of **5** with (*S*)-*O*-isopropylideneglycerol tosylate to give (*R*)-ketal **6** which could be readily deprotected under acid-catalyzed aqueous conditions to give the (*S*)-diol RO3201195[17] (Scheme 2) in accordance with Roche's original strategy.[13] Although the yield for these final steps was not high, it did provide sufficient compound for study in WS cells.

The ability of RO3201195 to inhibit p38α and JNK was tested in human hTERT-immortalized HCA2 cells by ELISA as described previously.[11,18] Kinase activity is detected using antibodies specific for phosphorylated (S73)HSP27 and antibodies that detect total levels of HSP27, the degree of activation being measured as the ratio of phospho-protein/total protein. In this system p38 activation by anisomycin activates MK2 that then phosphorylates HSP27. For JNK the phosphorylation status of the JNK substrate *c-jun* is monitored. In addition, the effect of RO3201195 on the p38 signalling pathway in WS cells was assessed by immunoblots.[11,18]

That RO3201195 can inhibit the p38α-signalling pathway is shown in Figure 2a and c. In control cells, low p38α activity is indicated by low p-HSP27 levels and a low p-HSP27/HSP27 ratio (DMSO columns). Anisomycin treatment activates p38α that increases p-HSP27 levels and the p-HSP27/HSP27 ratio (An columns). RO3201195 pre-treatment increasingly inhibits the anisomycin-induced activity of p38α, as indicated by the decreasing p-HSP27 levels and the p-HSP27/HSP27 ratio. Maximal inhibition is achieved between 2.5 and 10.0 μM RO3201195. The IC_{50} in this system is approximately 180–200 nM, similar to the reported IC_{50} for RO3201195 inhibition of p38α induced TNFα or IL-1β production in peripheral blood mononucleocytes of 500 nM and 400 nM,

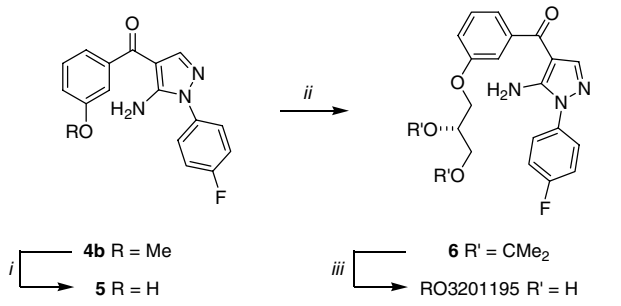
Table 1
Microwave-assisted conditions for the synthesis of **4a–n**

Entry	4	R^1	R^2	Yield%[a]				
				A	B	C	D	E
1[b]	**a**	H	Ph	41	75	95		
2[b]	**b**	OMe	4-Fluorophenyl	37	43	78	36	86[c]
3	**c**	OMe	4-Nitrophenyl		66		41	
4[b]	**d**	OMe	4-Methoxyphenyl		37			71
5[b]	**e**	OMe	*tert*-Bu					82
6[b]	**f**	OMe	*p*-Tolyl		36			58
7	**g**	OMe	Me		17		46	
8	**h**	OMe	H		53		77	
9	**i**	OMe	Pentafluorophenyl		71		78	
10[b]	**j**	OMe	2,4-Difluorophenyl		67	74		>98
11[b]	**k**	OMe	2-Fluorophenyl		9	72		90
12[b]	**l**	OMe	4-Bromophenyl		18	63		92
13	**m**	H	Pentafluorophenyl				89	
14	**n**	H	2,5-Dichlorophenyl				95	

[a] Isolated yield of **4** from one of the methods A–E. Method A: EtOH, reflux, 3 h (5 h for entry 2). Method B: microwaves, EtOH, 100 °C, 40 min (entry 1) or 120 °C, 20 min (entry 2) or 1 h. Method C: microwaves, EtOH, Et_3N, 100 °C (entries 1 and 2) or 120 °C (entries 10–12), 1 h. Method D: microwaves, EtOH, 140 °C, 20 min (entry 2) to 1 h (otherwise). Method E: microwaves, EtOH, Et_3N, 140 °C, 1 h. 'Microwaves' indicates microwave irradiation in a 10 mL Pyrex™ tube using a CEM Discover™ single-mode instrument at the specified temperature by moderating the initial magnetron power (100 W).

[b] The corresponding hydrazine hydrochloride was used.

[c] On scale up (124 mg of **3b**), irradiation at 110 °C for 1 h followed by 140 °C for 40 min avoided untoward pressure build up and gave **4b** (95 mg, 68%) suitable for elaboration to RO3201195.



Scheme 2. Synthesis of RO3201195 from pyrazol-4-yl ketone **4b**. Reagents and conditions: (i) BBr_3, CH_2Cl_2, 0 °C; RT, 2.5 h; H_2O (59%); (ii) (*S*)-L-α,β-isopropylideneglycerol-γ-tosylate, K_2CO_3, DMF, 80 °C, 24 h (9%); (iii) *p*-TsOH, MeOH, H_2O, 50 °C, 18 h (48%). RT, room temperature.

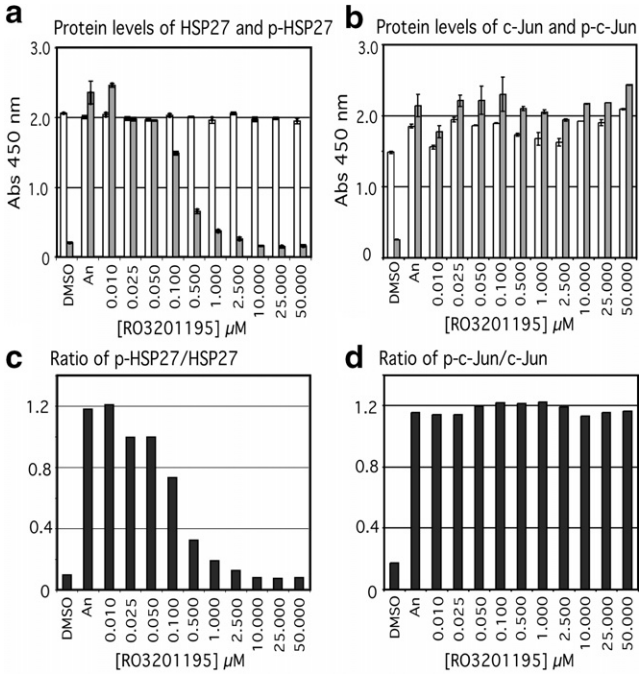


Figure 2. ELISA results for the effect of RO3201195 on p38α activity (a and c) and on JNK activity (b and d). For the upper panels, total protein is indicated by the white bars, and the phosphorylated protein by the dark grey bars. In the lower panel the ratio of phosphorylated protein to total protein is indicated by the black bars. DMSO are cells with only DMSO treatment, An are cells treated with anisomycin, and 0.010–50.000 are cells pre-treated with increasing concentrations of RO3201195 followed by treatment with anisomycin. p-HSP27 and p-*c-jun* are the phosphorylated forms of HSP27 and *c-jun*, respectively.

respectively, and the IC_{50} for RO3201195 inhibition of p38α-induced TNFα production in THP-1 cells of 250 nM.[13] Anisomycin treatment also activates JNK1 and JNK2 resulting in elevated levels of p-*c-jun* and an increased p-*c-jun*/*c-jun* ratio (Fig. 2b and d). RO3201195 is reported to inhibit JNK2 in an in vitro assay with a K_d of 5.0 μM;[13] however, RO3201195 pre-treatment of HCA2 cells does not prevent the anisomycin-induced phosphorylation of *c-jun* up to 50 μM. As the ELISA measures both JNK1 and JNK2 activity, these data show that RO3201195 does not inhibit total cellular JNK activity, and do not preclude the possibility that RO3201195 can inhibit either JNK1 or JNK2 alone.[13]

The ability of RO3201195 to inhibit the p38α-signalling pathway in hTERT-immortalized AG03141 WS cells was tested by immuno-detection of activated versions of p38α, MK2 and HSP27 on Western blots as described previously[11] (Fig. 3). In control WS cells, phosphorylated p38 is not detected; however, there is a

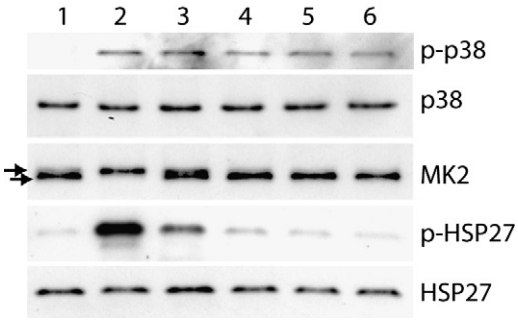


Figure 3. Effects of RO3201195 treatment on the activity of p38α in hTERT-immortalized WS cells. Lane 1, WS cells; lane 2, WS cells + anisomycin (A); lanes 3–5 WS cells + A + 2.5, 10.0 and 25.0 μM RO3201195; lane 6, WS cells + A + 2.5 μM SB203580. p-p38 and p-HSP27 are the phosphorylated forms of p38 and HSP27. With MK2 two bands are seen (arrows): the upper is activated (phosphorylated) MK2.

low level of activated p38 as assessed by the presence of activated MK2 (upper band in lane 1 of Fig. 3) and low levels of p-HSP27. Anisomycin treatment greatly increases the activation of p38α causing an increase in activated MK2 and p-HSP27 levels (lane 2). RO3201195 pre-treatment inhibits the anisomycin-induced activity of p38α, as indicated by the much-reduced levels of activated MK2 and p-HSP27 (lanes 3–5). As the level of activated MK2 is reduced in the RO3201195-treated cells compared to the control (compare lanes 1 and 5), RO3201195 not only inhibits the anisomycin-induced activity of p38α, but also the p38α activity that is believed to be caused by genomic stress in the WS cells.

In conclusion, the p38α MAPK inhibitor RO3201195 and other phenyl pyrazol-4-yl ketones can be prepared rapidly and efficiently from β-(anilino)-α-benzoylacrylonitriles by microwave irradiation with the corresponding hydrazine in ethanolic solvent. Further elaboration provided RO3201195, the inhibitory activity of which was confirmed in HCA2 and WS cells by ELISA and immunoblot assay, showing excellent selectivity for p38α MAPK over JNK. Given this selectivity profile, RO3201195 would appear to be ideal for further study to rescue premature senescence and the accelerated ageing of WS cells in culture. These studies are now underway in our laboratories and will be reported in due course.

Acknowledgements

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16. In a typical procedure, a mixture of 3-methoxy-2-benzoyl-3-phenyl-aminoacrylonitrile (**3b**) (20 mg, 0.07 mmol) and *p*-fluorophenylhydrazine hydrochlororide (12 mg, 0.07 mmol) and triethylamine (11 μL, 0.08 mmol) in ethanol (0.8 mL) was irradiated in a sealed tube at 140 °C for 1 h using a CEM Discover™ single-mode microwave synthesizer, by moderating the initial microwave power (100 W). After cooling in a stream of compressed air, the mixture was evaporated in vacuo and purified by column chromatography on silica, eluting with petroleum ether–EtOAc (6:4), to give **4b** (19 mg, 86%).
17. 5-Amino-1-(4-fluorophenyl)-4-{3-[2(*S*),3-dihydro-xypropoxy]benzoyl}pyrazole (RO3201195) [R_f 0.27 (CH_2Cl_2–MeOH, 9:1)] was obtained as a colourless solid, mp 75 °C (MeOH) (Found: MH^+, 372.1354. $C_{19}H_{19}N_3O_4F$ requires [MH^+], 372.1352); $[\alpha]_D^{22}$ + 4.8 (*c* 0.23, MeOH); IR (KBr)/cm^{-1} ν_{max} 3404, 2928, 1623, 1600, 1576, 1537, 1513, 1503, 1440, 1313, 1288, 1223, 1049, 932, 843, 767; ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 3.43–3.48 (2H, m, C*H*$_2$OH), 3.78–3.85 (1H, m, C*H*OH), 3.90–3.96 (1H, m, OCH*H*CHOH), 4.05–4.11 (1H, m, OC*H*HCHOH), 4.72 (1H, t, *J* 5.7 Hz, *H*OCH$_2$), 5.02 (1H, d, *J* 4.8 Hz, *H*OCH), 7.13–7.20 (3H, NH_2 and 4′-Ar*H*), 7.24 (1H, s, 2′-Ar*H*), 7.31–7.35 (1H, m, 5′-Ar*H*), 7.37–7.46 (3H), 7.58–7.64 (2H), 7.80 (1H, s, 3-H); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 62.6 (CH_2), 69.7 (CH_2), 69.9 (CH), 103.4 (C), 113.2 (CH), 116.2 (d, $^2J_{C-F}$ 21.2 Hz, CH), 117.8 (CH), 120.1 (CH), 126.4 (d, $^3J_{C-F}$ 9.1 Hz, CH), 129.7 (CH), 133.7 (d, $^4J_{C-F}$ 3.0 Hz, C), 140.9 (C), 141.5 (CH), 151.2 (C), 158.7 (C), 161.1 (d, $^1J_{C-F}$ 245.9 Hz, C), 187.6 (C); *m/z* (APcI) 372 (MH^+, 100%).
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