



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 p38 α MAPK over the related stress-activated kinase JNK.

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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 p38 α ,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 p38 α 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 p38 α MAPK.⁹ Subsequently we showed that alternative p38 inhibitors with improved kinase selectivity, including BIRB 796¹⁰ 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 JNK1 α 1 and JNK2 α 1 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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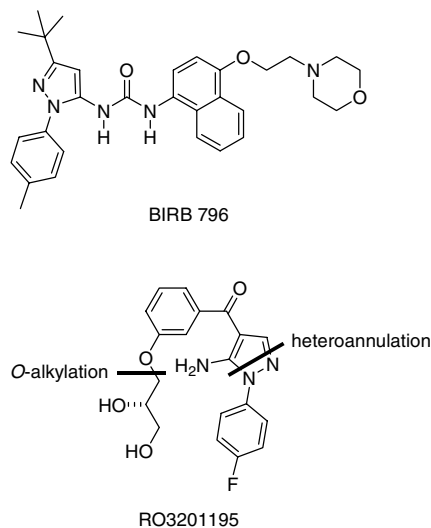


Figure 1. The p38 MAPK inhibitors BIRB 796 and RO3201195, the latter showing our disconnection strategy.

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 regioselective heterocyclocondensation of the corresponding hydrazine and a β -anilino- α -benzoylacrylonitrile formed by Knoevenagel-type condensation. *O*-Alkylation installs the propoxy 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 pyrazoles¹⁴ 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 conduc-

tive heating and microwave irradiation in a sealed vessel at 100–140 °C in the presence or absence of Et₃N (Table 1).¹⁶

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 Et₃N 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*-isopropylidenediglycerol tosylate to give (*R*)-ketal **6** which could be readily deprotected under acid-catalyzed aqueous conditions to give the (*S*)-diol RO3201195¹⁷ (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 p38 α and JNK was tested in human hTERT-immortalized HCA2 cells 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 p38 α -signalling pathway is shown in Figure 2a and c. In control cells, low p38 α activity is indicated by low p-HSP27 levels and a low p-HSP27/HSP27 ratio (DMSO columns). Anisomycin treatment activates p38 α that increases p-HSP27 levels and the p-HSP27/HSP27 ratio (An columns). RO3201195 pre-treatment increasingly inhibits the anisomycin-induced activity of p38 α , as indicated by the decreasing p-HSP27 levels and the p-HSP27/HSP27 ratio. Maximal inhibition is achieved between 2.5 and 10.0 μ M RO3201195. The IC₅₀ in this system is approximately 180–200 nM, similar to the reported IC₅₀ for RO3201195 inhibition of p38 α induced TNF α or IL-1 β production in peripheral blood mononucleocytes of 500 nM and 400 nM,

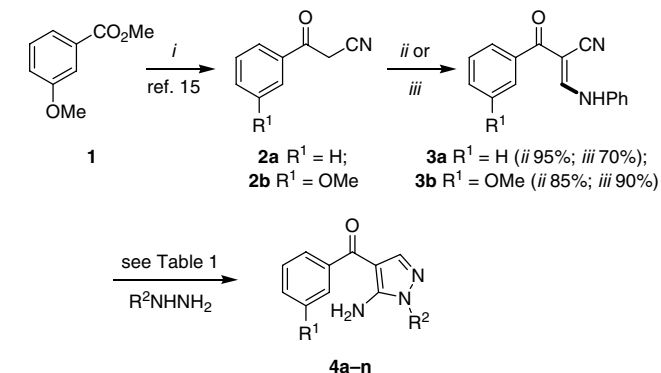
Table 1
Microwave-assisted conditions for the synthesis of **4a–n**

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	<i>tert</i> -Bu					82
6 ^b	f	OMe	<i>p</i> -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, Et₃N, 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, Et₃N, 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 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.

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