

Synthesis and in vivo activity of MK2 and MK2 substrate-selective p38 α ^{MAPK} inhibitors in Werner syndrome cells

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Abstract—A benzopyranopyridine inhibitor of mitogen-activated protein kinase-activated protein kinase 2 (MK2) is prepared rapidly and efficiently in one step using microwave dielectric heating, whereas a substrate-selective p38 MAPK inhibitor was prepared using conventional heating techniques. The former had MK2 inhibitory activity above 2.5 μ M concentration, whereas the latter showed no MK2 inhibition at 10 μ M. However, rather than rescuing the reduced cellular growth rate and aged morphology of hTERT-immortalised WS dermal fibroblasts, both induce a state resembling stress-induced cellular senescence, suggesting that these inhibitors may have limited therapeutic use.

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Werner syndrome (WS) is a genetic disorder resulting from mutation in the RecQ helicase-encoding *WRN* gene.¹ WS individuals show premature onset of many clinical features of old age, show early susceptibility to a number of major age-related diseases and have a greatly abbreviated median life expectancy (47 years) as a consequence of malignancy and myocardial infarction.¹ WS is widely used as a model disease to investigate normal human ageing processes.² Several observations suggest that accelerated cellular senescence may underlie several features of accelerated ageing in WS, as cells from a dividing tissue (e.g., skin fibroblasts) that shows premature in vivo ageing also show accelerated in vitro ageing,^{3,4} whereas cells from a dividing tissue (e.g., T cells) that does not manifest premature ageing in vivo do not.⁵ Whether cellular senescence plays a role in organismal ageing has been hotly disputed;⁶ however, recent observations demonstrate that senescent fibroblasts accumulate in the skin during ageing.⁷ Normal and WS fibroblasts use telomere erosion as a cell division ‘counter’ and senesce with short telomeres.⁸ How-

ever, unlike normal fibroblasts, WS fibroblasts show activation of the stress-associated MAPK p38 α (*herein* p38), and studies using the p38 inhibitor SB203580 rescued the features of accelerated replicative decline and allowed the WS cells to attain a normal lifespan.⁴ These data suggest that WS cells show premature senescence via activation of a ‘stress signal’, possibly due to the stalled DNA replication forks that are characteristic of cells deficient in the WRNp helicase,⁹ leading to a phenomenon known as replication stress.¹⁰ This replication stress synergises with telomere erosion to limit WS cell lifespan. If accelerated ageing in WS is related to p38 activation and to accelerated cell ageing, then WS provides a powerful model to link cellular signalling events to the ageing of mitotic tissues in vivo, and also may provide opportunities for therapeutic intervention. However, p38 regulates many cellular processes making p38 inhibitors problematical for therapeutic use due to the possibilities of deleterious side effects.¹¹

Many young WS cells are enlarged with prominent F-actin stress fibres and resemble aged normal cells.⁴ The p38 pathway is involved in stress fibre production via activation of MK2 that then phosphorylates HSP27.¹² In addition, recent data suggest that MK2 acts as a checkpoint kinase that can lead to cell-cycle arrest.¹³ Moreover, MK2 activity up-regulates the expression of

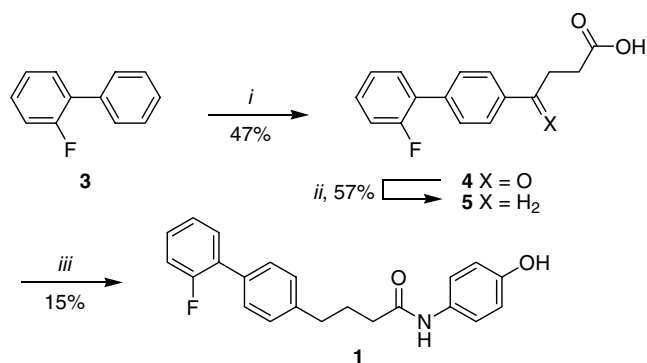
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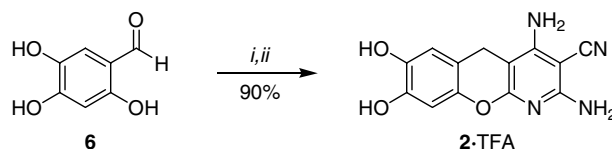
inflammatory pathways,¹⁴ and WS is associated with inflammatory diseases.¹⁵ These data suggest the possibility that MK2 may be involved in the phenotypic characteristics seen in WS. If so, then therapeutic targeting of MK2 may help surmount complications in the use of p38 inhibitors, as the number of pathways inhibited will be reduced. The recent disclosure of small molecule inhibitors of MK2 makes this approach possible.^{16,17} We synthesized two different MK2 inhibitors (see Schemes 1 and 2). The first biphenyl **1** is a substrate specific inhibitor that actively prevents the activation of MK2 by p38, while leaving p38 activation of other targets alone (e.g., ATF2).¹⁶ The second, diaminocyanopyridine **2**, is an ATP-competitive inhibitor of MK2 that is effective in attenuating TNF α production in cellular assays with established *in vivo* activity.¹⁷

Our route to the substrate-selective inhibitor **1** involved the Friedel–Crafts acylation of fluorobiphenyl **3** using succinic anhydride/ AlCl_3 , followed by Clemmensen reduction of ketoacid **4** to give carboxylic acid **5**, suitable for amide formation with 4-aminophenol (Scheme 1). Benzotriazol-1-yloxytriethylphosphonium hexafluorophosphate (pyBOP)-mediated coupling gave the inhibitor **1**,¹⁸ albeit it in very poor (15%) yield. This unoptimized process, undoubtedly a consequence of low aniline reactivity, did provide sufficient quantity on small scale (0.2 mmol) for biological evaluation.

The synthesis of inhibitor **2** from salicylaldehyde **6** by condensation with malonitrile dimer in EtOH–AcOH was first investigated under traditional conductive heating methods at reflux for 21 h, followed by evaporation, addition of trifluoroacetic acid (TFA) and reduction with triethylsilane. However, under these conditions,¹⁷ the TFA salt of **2** did not precipitate and only unreacted starting material was obtained so recourse was made to more forcing conditions. Microwave irradiation at 140 °C for 1 h in a sealed tube using a single-mode microwave synthesizer (CEM DiscoverTM), followed by triethylsilane reduction in TFA, precipitated the TFA salt of **2**¹⁹ in 90% yield (Scheme 2), which could be liberated as the free base by treatment with ethanolic ammonia.



Scheme 1. Synthesis of MK2 substrate-selective p38 inhibitor **1**. Reagents and conditions: (i) succinic anhydride, AlCl_3 , $\text{ClCH}_2\text{CH}_2\text{Cl}$, 24 h; (ii) Zn, HgCl_2 , HCl (aq), 5 min; then **4**, toluene, H_2O , reflux, 30 h; (iii) PyBOP, dry CH_2Cl_2 , 0 °C; 4-aminophenol, Et_3N , rt, 24 h.



Scheme 2. Synthesis of MK2 inhibitor **2**. Reagents and conditions: (i) malonitrile dimer, EtOH–AcOH (1:1), microwaves, 140 °C (initial power 120 W, which was moderated to maintain a constant reaction temperature), 1 h; (ii) Et_3SiH , TFA, 0 °C, 4 h.

MK2 inhibitory activity was assessed by the ability to prevent the anisomycin-induced phosphorylation of HSP27 in human hTERT-immortalised HCA2 cells using an ELISA system as previously described.²⁰ In addition, the inhibitors were tested for their ability to modify the growth rate and morphology of immortalised HCA2 and WS cells using daily replaced drug supplemented growth medium as previously described.⁴ Finally, their effects on p38 activation were assessed by immunoblots.²⁰ For inhibitor **2**, the same results were found using either the free base or the TFA salt.

Inhibitor **1** prevents the ability of p38 to activate MK2 using an *in vitro* kinase assay with a K_i^{app} of 330 nM, by binding in the vicinity of the p38 active site causing subtle structural perturbations that result in suboptimal positioning of substrates and cofactors.¹⁶ However, inhibitor **1** at 10 μM has, at best, only a small effect on the anisomycin-induced phosphorylation of HSP27 in HCA2 cells (Fig. 1a), or in WS cells (Fig. 1c). SB203580, a p38 inhibitor, prevents HSP27 phosphorylation in WS cells under similar conditions (Fig. 1c). As the reported inhibition of MK2 at 10 μM is an *in vitro* assay with purified proteins,¹⁶ it is possible that we find little inhibition of MK2 due to the cellular context. Such effects have been seen for other kinase inhibitors.²² Inhibitor **1** and SB203580 have only a small effect on the anisomycin-induced activation of p38 in WS cells as expected, as these inhibitors act to affect p38 activity, not its activation. Continuous treatment of WS cells with inhibitor **1** above 200 nM resulted in complete cessation of growth (Fig. 1b).

In contrast, inhibitor **2** prevents the anisomycin-induced phosphorylation of HSP27 above 2.5 μM in HCA2 cells, with the maximal inhibition achieved at 25 μM (Fig. 2a). The IC_{50} seems to be between 2.5 and 10 μM , higher than the reported IC_{50} of 0.92 μM to attenuate LPS-induced TNF α production in U937 cells.¹⁷ However, this agrees with data showing that inhibitor **2** prevents the arsenite-induced phosphorylation of HSP27 in mouse embryonic fibroblasts and HeLa cells at 15 μM .²¹ Inhibitor **2** at 25 μM has no effect on the activation of p38 in control HCA2 cells, but may accentuate p38 activation in anisomycin-treated cells (Fig. 2b). Additionally, inhibitor **2** does not prevent the p38 phosphorylation of MK2, whereas SB203580 does. Similar effects were seen in WS cells (Fig. 3a). Continuous treatment of HCA2 and WS cells with inhibitor **2** at concentrations above 2.5 μM caused a complete cessation of growth (Fig. 3c).

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