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Synthesis and in vivo activity of MK2 and MK2 substrate-selective p38α^{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 μM concentration, whereas the latter showed no MK2 inhibition at 10 μM. 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.

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] However, unlike normal fibroblasts, WS fibroblasts show activation of the stress-associated MAPK p38α (*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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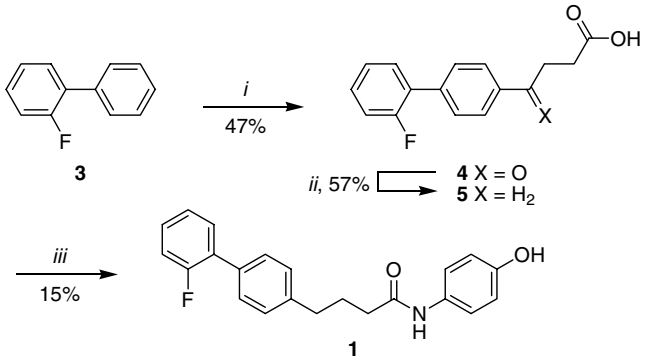
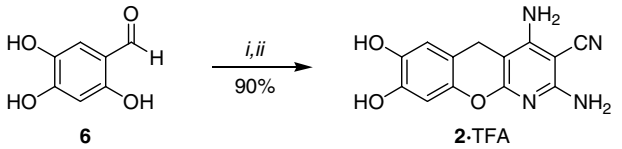
inflammatory pathways,[14] and WS is associated with inflammatory diseases.[15] These data suggest the possibility that MK2 may be involved in the phenotypic characteristics seen in WS. If so, then therapeutic targeting of MK2 may help surmount complications in the use of p38 inhibitors, as the number 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 TNFα 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-yloxytripyrrolidino-phosphonium 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 Discover™), 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, $AlCl_3$, $ClCH_2CH_2Cl$, 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 TNFα 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).

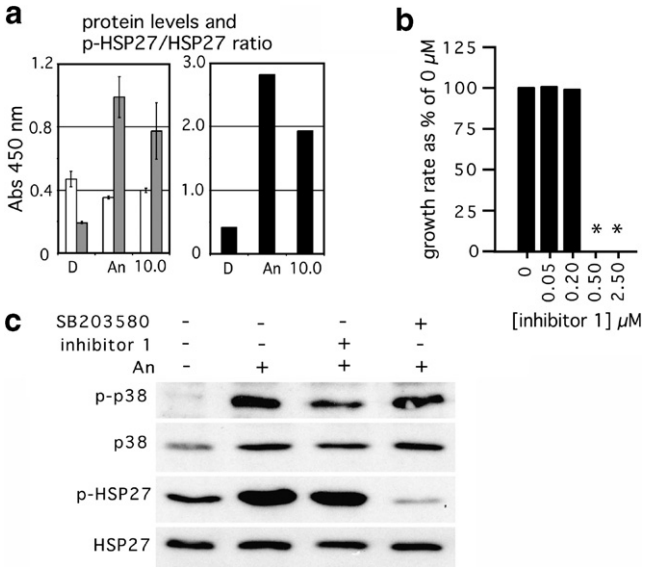


Figure 1. (a) Effects of inhibitor **1** on MK2 activity in HCA2 cells assessed by ELISA. White bars indicate total HSP27, dark grey bars p-HSP27 and black bars the p-HSP27/HSP27 ratio. D are DMSO-treated cells, An are anisomycin-treated cells, 10.0 are cells pre-treated with 10 μM inhibitor **1** followed by anisomycin treatment. (b) Effects of inhibitor **1** on the growth rate of WS cells: *zero growth rate. Growth rates are measured as population doublings (PD) per day with cells grown in standard EMEM in tissue culture flasks: PD = log(Nf/Ni)/log2 where Nf is the number of cells counted and Ni is the number of cells seeded. (c) Immunoblot showing effect of inhibitor **1** (10 μM) on the anisomycin-induced activity of the p38 pathway in WS cells. SB203580 (2.5 μM) is used as a control. p-HSP27 and p-p38 are the phosphorylated forms of HSP27 and p38.

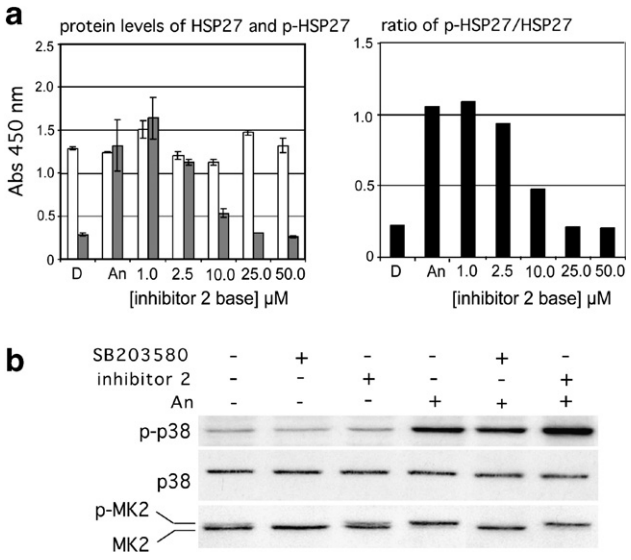


Figure 2. (a) ELISA results for the effects of inhibitor **2** base on MK2 activity in HCA2 cells. The key is as for Figure 1a. 1.0–50.0 are cells pre-treated with inhibitor **2** followed by anisomycin treatment. (b) Effects of inhibitor **2** (25 μM) and SB203580 (2.5 μM) on the activation of p38 and MK2. For MK2 the upper band is phosphorylated MK2 (p-MK2).

The WS cells used in this work have been immortalised using human telomerase expression;[4] however, these cells retain the slow growth and stressed morphology

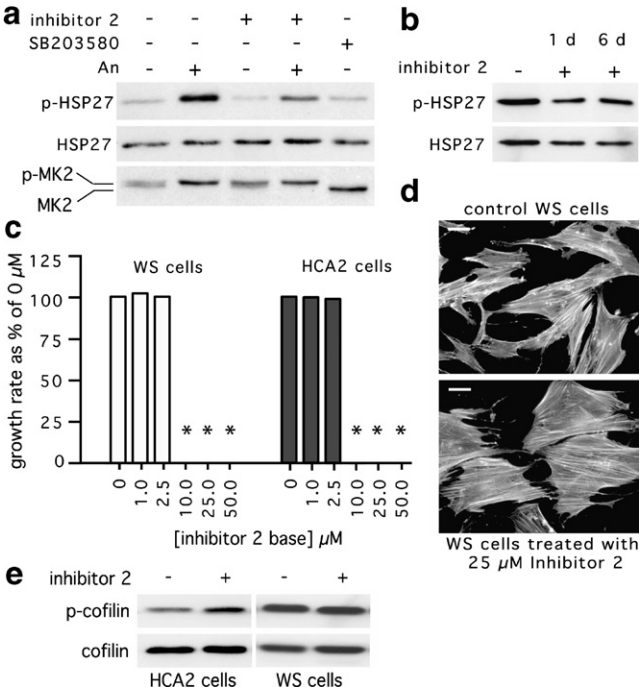


Figure 3. (a) Effects of inhibitor **2** (25 μM) and SB203580 (2.5 μM) on the activation of MK2 and HSP27 in WS cells. (b) Effects of inhibitor **2** (25 μM) on HSP27 phosphorylation in WS cells over 6 d. (c) Growth rate of WS and HCA2 cells in the presence of increasing concentrations of inhibitor **2**: *zero growth rate. (d) WS cells stained with phalloidin-FITC to visualize F-actin. Bar = 50 μm. (e) Effects of 25 μM inhibitor **2** on activation of cofilin.

seen in primary WS cells, in that many are enlarged with prominent F-actin stress fibres (Fig. 3d). These fibres are associated with elevated p-HSP27 levels that are reduced by SB203580 (Fig. 1c) and inhibitor **2** (Fig. 3b). Treatment with inhibitor **2** at 10 μM or above accentuated this phenotype, with more cells having increased levels of stress fibres, indeed they now resembled senescent cells (Fig. 3d). Similar effects are seen with inhibitor **1** (not shown). Inhibitor **2** shows similar effects on HCA2 cells (not shown).

The growth arrest caused by these inhibitors is unlikely to be related to MK2 inhibition, as it appears to occur at inhibitor **2** levels insufficient to inhibit MK2 by more than 50%, and inhibitor **1** shows no MK2 inhibitory activity. Likewise, the increased levels of stress fibres are unlikely to result from MK2 activity, as inhibitor **2** at 25 μM results in decreased p-HSP27 levels in WS cells that are maintained over 6 days (Fig. 3b). These inhibitors may be targeting a second kinase, although as these inhibitors work in very different ways, the probability that they would hit the same off-target seems small. One possible off-target may be the stress kinase JNK; however, we have shown that these inhibitors have no effects on JNK in a cell-based assay (not shown). Alternatively, these effects may simply be due to cell toxicity. If so, then unlike other cell toxic chemicals such as arsenite, their effects are not transduced via the p38- or JNK-signalling pathways. A further possibility is that these inhibitors may increase F-actin stress fibre production by activating the lim kinase (LIMK) pathway

resulting in phosphorylation of the actin depolymerising protein cofilin.[23] It has previously been shown that cofilin is phosphorylated in young primary WS cells and also in senescent WS and normal cells.[4] Indeed, inhibitor **2** treatment increases the level of phosphorylated cofilin in HCA2 cells, although not in WS cells (Fig. 3e); however, cofilin may already be maximally activated in the WS cells. Finally, an ironic conclusion would be that, as MK2 inhibition in WS cells does not duplicate what is seen using p38 inhibitors,[4] these data may actually suggest that MK2 is not involved in the WS phenotype, although this is speculative. The development of new MK2 inhibitors with much lower IC_{50} values in cell-based systems would be needed to show this. In conclusion, two MK2 inhibitors have been prepared and tested for their effects on WS cells. The herein reported effects of these inhibitors suggest that despite the observed activity of inhibitor **2** in a 2-h rat LPS challenge assay (20 mpk, IP, 68% inhibition),[17] they would be unsuitable for long-term therapeutic use due to toxicity issues and the possibility of deleterious side effects.

Acknowledgments

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18. Inhibitor **1** was obtained as a colourless solid, mp 128–130 °C (found: MH^+ 350.1548, $C_{22}H_{21}FNO_2$ [MH^+] requires 350.1551); IR (KBr) (ν/cm^{-1}) 3317, 2965, 2816, 1654, 1514, 1350, 1230, 1100, 880; ^{1}H NMR (400 MHz, $CDCl_3$) (δ/ppm) 7.44–7.40 (2H, m, ArH), 7.39–7.32 (1H, m, ArH), 7.30–7.18 (7H, ArH), 7.16–7.04 (2H, m, ArH), 6.92 (1H, br s, NH), 6.73 (2H, d, *J* 8 Hz, ArH), 4.95 (1H, br s, OH), 2.70 (2H, t, *J* 7.3, CH_2), 2.29 (2H, t, *J* 7.4, CH_2), 2.04 (2H, tt, *J* 7.3, 7.4, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) (δ/ppm) 170.8 (C), 159.7 (d, $^1J_{C-F}$ 247 Hz, C), 152.5 (C), 140.8 (C), 133.6 (C), 130.7 (C), 130.6 (d, $^3J_{C-F}$ 3.8 Hz, CH), 129.1 (d, $^4J_{C-F}$ 3.1 Hz, CH), 128.8 (d, $^3J_{C-F}$ 7.9 Hz, CH), 128.7 (CH), 128.6 (C), 124.4 (d, $^4J_{C-F}$ 3.7 Hz, CH), 122.1 (CH), 116.1 (d, $^2J_{C-F}$ 22.8 Hz, CH), 115.6 (CH), 36.5 (CH_2), 34.7 (CH_2), 26.8 (CH_2); MS (APcI) *m*/*z* 350 (MH^+, 100%).
19. Inhibitor **2**·TFA was obtained as an orange solid, mp >290 °C (found: MH^+ 271.0824, $C_{13}H_{11}N_4O_3$ [MH^+] requires 271.0826); IR (KBr) (ν/cm^{-1}) 3480, 3397, 3359, 3258, 3223, 3112, 3067, 2926, 2859, 2230, 1675, 1652, 1559, 1521, 1505, 1457, 1444, 1394, 1324, 1287, 1158, 1132; ^{1}H NMR (400 MHz, d_6-DMSO) (δ/ppm) 9.85–8.00 (2H, br s, 2OH), 7.30–6.60 (2H, br s, NH_2), 7.10 (2H, br s, NH_2), 6.64 (1H, s, 6H), 6.44 (1H, s, 9H), 3.49 (2H, s, 5H); ^{13}C NMR (125 MHz, d_6-DMSO) (δ/ppm) (one C not observed) 158.9 (C), 158.5 (C), 145.3 (C), 142.9 (C), 142.3 (C), 116.0 (C), 115.0 (CH), 108.7 (C), 104.0 (CH), 86.9 (C), 70.1 (C), 22.3 (CH_2); MS (ES) *m*/*z* 271 (MH^+, 100%).
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