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Rapid synthesis of VX-745: p38 MAP kinase inhibition in Werner syndrome cells

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Abstract—The p38 mitogen-activated protein kinase inhibitor VX-745 is prepared rapidly and efficiently in a four-step sequence using a combination of conductive heating and microwave-mediated steps. Its inhibitory activity was confirmed in hTERT immortalized HCA2 and WS dermal fibroblasts at 0.5–1.0 μM concentration by ELISA and immunoblot assay, and displays excellent kinase selectivity over the related stress-activated kinase JNK.

Werner syndrome (WS) is a rare genetic disorder resulting from mutation in the RecQ helicase-encoding *WRN* gene.[1] Mutations in *WRN* are null mutations at the functional level resulting in lack of production of WRN protein. 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] Thus, WS is widely used as a model disease to investigate normal human ageing processes.[2]

Normal human cells divide a finite number of times in culture after which they enter a state of permanent growth arrest termed senescence. This finite proliferative capacity may be causal in normal ageing, as senescent cells are known to accumulate in ageing tissue,[3] and they display deleterious biochemical features, such as a pro-inflammatory phenotype and an altered ability to remodel the extracellular matrix, suggesting a link between replicative senescence and tissue degeneration.[4] The mechanisms by which replicative senescence occurs are not fully understood, but it offers a new way to approach the cause and prevention of age-related degenerations, by understanding and modulating the basic process of ageing itself. WS fibroblasts are studied because they show accelerated ageing in vitro[5] that may underlie some of the in vivo accelerated ageing that occurs in WS.[2,4] Our model system, comprising cultured primary fibroblast cells from WS individuals, provides a powerful means to understand the ageing of mitotic tissue in vivo and links it with cellular signalling events. Normal and WS fibroblasts use telomere erosion as a cell division 'counter';[6] however, telomere-driven senescence synergizes with a telomere-erosion-independent mechanism in WS.[7] Unlike normal fibroblasts, proliferating WS cells contain high levels of phosphorylated p38α,[8] a stress-associated mitogen-activated protein kinase (MAPK). WS cells show many of the characteristics of cells growing under 'replication stress',[9] with a slow growth rate, enlarged morphology and prominent actin stress fibres. Thus, the accelerated replicative decline in WS may be caused by a 'stress signal'[10] from the stalled replication forks that are characteristic of cells deficient in the WRNp helicase.[11]

If true, then blocking the p38α response through the use of chemical inhibitors has the potential to modulate the cellular and ultimately clinical pathology of WS. Initial studies using the prototypical p38α inhibitor SB203580 (Fig. 1) in WS cells rescued the features of accelerated replicative decline, including growth rate and cell morphology,[8] suggesting that the abbreviated life span of WS cells was due to a stress-induced growth arrest mediated by p38α MAPK. However SB203580, whilst useful

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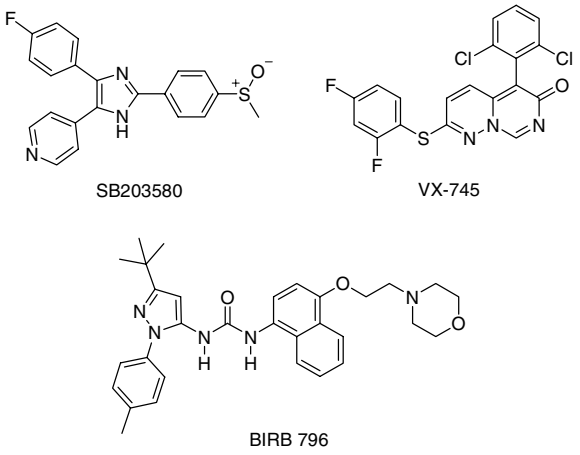
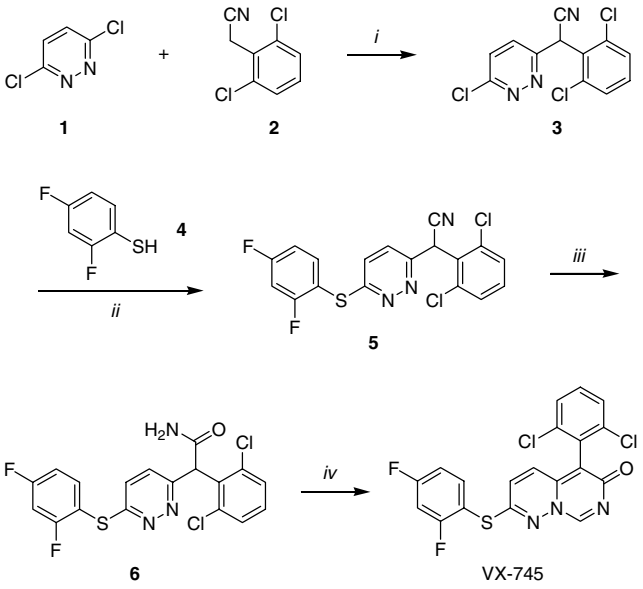


Figure 1. p38 MAPK inhibitors for evaluation in WS cells.

for research applications, lacks the kinase selectivity profile that is desirable for a therapeutic agent. Our recent report on a route to BIRB 796, a p38α inhibitor with a different mode of action to SB203580,[12] provided an alternative inhibitor for study.[13] However, BIRB 796 appears to have some similarities in cross-kinase specificity to SB203580, in particular with regard to the stress-activated JNK kinases.[14] As young WS cells show activation of the p46 forms of JNK, facile access to another chemotype without JNK inhibitory activity is required to corroborate these findings.

In 1999 Vertex Pharmaceuticals disclosed a new class of p38α ATP competitive inhibitors including the clinical candidate VX-745.[15] This compound displayed potent activity at 5.0 nM concentration and 1000-fold selectivity over closely related kinases, including ERK1, JNK1-3 and MK2, adopting a linear rather than teardrop or extended binding mode.[16] With such an exquisite selectivity profile, complementary binding motif and clinical efficacy in rheumatoid arthritis patients,[17] this inhibitor became a compelling compound of study in WS cells. However although the synthesis of VX-745 has been described,[15] the efficiency of some of the steps is very low and the methods have been reported to be poorly reproducible.[18] In this manuscript we describe a rapid and efficient route to VX-745 using a combination of microwave irradiation and conductive heating techniques and its evaluation as a p38 MAPK inhibitor in WS cells.

A natural starting point for our synthesis (Scheme 1) was 3,6-dichloropyridazine (**1**), in common with previous approaches,[15,16,18] as the selective sequential elaboration of this difunctionalized building block is known. Reaction with (dichlorophenyl)acetonitrile **2**, on deprotonation with sodium amide or sodium hydride, does give pyridizinylacetonitrile **3**, but in only 28%[15,16] or 43%[18] yield, respectively, the latter of which was reproduced exactly in our laboratories. By switching to potassium *tert*-butoxide the efficiency of reaction was improved to 73%, but this was only after exhaustive chromatographic purification to remove unreacted pyridazine **1**, impeding considerably the facility of the process. By carrying out the reaction under microwave



Scheme 1. Synthesis of VX-745. Reagents and conditions: (i) KO^{t}Bu, PhMe, microwaves, 120 °C (initial power 150 W), 1.5 h (62%); (ii) see Table 1; (iii) concd H_2SO_4, microwaves, 100 °C (initial power 100 W), 30 min (68%); (iv) *N*,*N*-dimethylformamide dimethyl acetal, PhMe, 100 °C, 2 h; then room temperature, 18 h (87%).

irradiation in toluene at 120 °C for 1.5 h, conversion was complete and the purification of **3** was simplified considerably, albeit with a minor reduction in yield. Subsequent reaction of **3** with sodium 2,4-difluorothiophenoxide, prepared from thiophenol **4** by treatment with NaH, at room temperature in DMF (shown to improve the solubility of **3**),[18] provided thiopyridazine **5** in 57% yield. Once again contamination by unreacted starting materials complicated the purification process considerably and so a range of alternative conditions were investigated (Table 1). Traditional conductive heating conditions, palladium-catalyzed or copper-mediated Ullmann-type couplings,[19] gave the product **5** in improved yields (entries 1, 4 and 6) although only after lengthy reaction times. Out of the microwave-assisted procedures (entries 3, 5 and 7), only the use of the palladium (II) *N*-heterocyclic-carbene (NHC) catalyst PEPPSI™-IPr (Pyridine-Enhanced Precatalyst Preparation Stabilization and Initiation)[20] gave a rapid and efficient route to **5** (entry 5). This particular protocol demonstrated several attractive features, which included rapid reaction time, low catalyst loading and a considerably simplified purification process.

From thiopyridazine **5**, hydrolysis of the nitrile group to the corresponding amide **6** could be effected in concentrated sulfuric acid at 100 °C for 2 h by conductive heating or by microwave irradiation at 100 °C for 30 min, in 63% or 68% yield, respectively. Finally, heating **6** with *N*,*N*-dimethylformamide dimethyl acetal at 100 °C in toluene for 2 h, followed by an overnight stir at room temperature, gave the pyrimido[1,6-*b*]pyridazinone motif of VX-745 in yields that ranged between 87% and >98%. Overall our rapid route uses microwave irradiation to dramatically accelerate three out of the four steps and represents a reproducible and efficient route to VX-745 in >98% purity[21] and 28% overall yield.

Table 1. Reagents and conditions for the synthesis of sulfide **5** step (ii)

Entry	Conditions[a]	Yield %[b]
1	KO^{t}Bu, Pd(OAc)$_2$ (5 mol %), (*S*)-TolBINAP, PhMe, reflux, 16 h	73
2	NaO^{t}Bu, Pd(OAc)$_2$ (5 mol %), DPPF, PhMe, reflux, 16 h	31
3	NaO^{t}Bu, Pd(OAc)$_2$ (5 mol %), DPPF, PhMe, microwaves (120 W), 150 °C, 1 h	0
4	PEPPSI™-IPr (2 mol %), NaO^{t}Bu, PhMe, reflux, 16 h	75
5	PEPPSI™-IPr (2 mol %), NaO^{t}Bu, PhMe, microwaves (120 W), 150 °C, 1 h	75
6	CuI (10 mol %), Et$_3$N, dioxane, 90 °C, 16 h	82
7	CuI (10 mol %), Et$_3$N, dioxane, microwaves, 100 °C, 3 h	24

[a] Microwaves indicates irradiation in a CEM Discover™ at the given temperature through moderation of initial microwave power (given in parentheses); PEPPSI™-IPr, [1,3-bis(2,6-diisopropylphenyl)imidazol-2-ylidene](3-chloropyridyl)palladium(II) dichloride; DPPF, 1,1′-ferrocene-bis(diphenylphosphine); (*S*)-TolBINAP, (*S*)-(−)-2,2′-bis(di-*p*-tolylphosphino)-1,1′-binaphthyl.
[b] Isolated yield of **5** after purification on silica.

That VX-745 can inhibit the p38α and JNK pathways was tested in human immortalised HCA2 cells using an ELISA system (Cell Signalling, NEB, UK). Cells were seeded in 100-mm dishes in EMEM and incubated at 37 °C for 48 h as previously described.[12] The medium was supplemented with VX-745 at final concentrations from 10 nM to 50 μM and the cells incubated for a further 2 h. Anisomycin was added to the medium at 30 μM and the cells harvested 45 min later. Samples using DMSO only, and DMSO plus anisomycin, were used as controls. Cells were harvested, proteins isolated and the ELISAs carried out according to the manufacturer's instructions. Kinase activity was detected using antibodies specific for the phosphorylated forms of HSP27 and *c-jun*, and antibodies that detect the total levels of HSP27 and *c-jun*, the degree of activation being measured as the ratio of phospho-protein/total protein. In this system activation of p38α by anisomycin activates MK2 that then phosphorylates the small heat-shock protein HSP27. As MK2 is the major HSP27 kinase, the activity of p38α can be assessed by the phosphorylation status of HSP27.[22] The results are shown in Figure 2 (a and c). In control cells there is a low level of p38α activation, as indicated by a low level of p-HSP27 and a low p-HSP27/HSP27 ratio (DMSO columns). Anisomycin treatment greatly increases the activation of p38α causing an increase in the p-HSP27 level and the p-HSP27/HSP27 ratio ('An' columns). Pre-treatment of cells with increasing concentrations of VX-745 increasingly inhibits the anisomycin-induced activity of p38α, as indicated by the decreasing levels of p-HSP27 and the p-HSP27/HSP27 ratio. Even at 10 nM VX-745 inhibits p38α to some extent, and maximal inhibition is achieved at 500 nM, with the level of p-HSP27 and the p-HSP27/HSP27 ratio now reduced to the basal level seen in the control. Interestingly, VX-745 at 50 nM appears to increase levels of HSP27 phosphorylation compared to 25 nM. It is possible that at concentrations above 25 nM VX-745 can activate the p38 pathway to some extent. However, at higher concentrations any activation effect is clearly countered by the inhibitory action of VX-745. The concentration of VX-745 that has a 50% inhibitory effect is between 10 and 100 nM, similar to the previously reported IC$_{50}$ of 56 nM for VX-745 inhibition of TNFα-stimulated p38α activity in peripheral blood mononucleocytes.[13] Anisomycin treatment also activates JNK (monitored

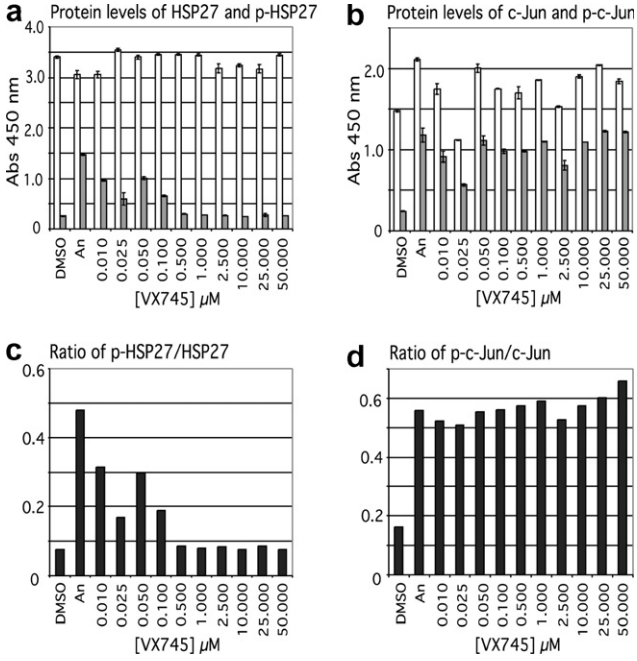


Figure 2. ELISA results for the effect of VX-745 on p38α activity (a, c) and on JNK activity (b, d). For the upper panels, the level of total protein is indicated by the white bars, and the level of the phosphorylated protein by the dark grey bars. In the lower panel the ratio of phosphorylated protein 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 VX-745 followed by treatment with anisomycin. p-HSP27 and p-*c-jun* are the phosphorylated forms of HSP27 and *c-jun*, respectively.

by the phosphorylation status of the JNK substrate *c-jun*) resulting in elevated levels of p-*c-jun* and an increased p-*c-jun*/*c-jun* ratio (Fig. 2b and d). However, pre-treatment of the cells with increasing concentrations of VX-745 has no effect on the anisomycin-induced activity of JNK towards *c-jun*, thus confirming that VX-745 is not a JNK inhibitor.[15]

The ability of VX-745 to inhibit the p38α signalling pathway in hTERT-immortalised AG03141 WS dermal cells was tested by immunoblot detection of activated versions of p38α and HSP27 as described previously[12] (Fig. 3). In control WS cells there was a low level of

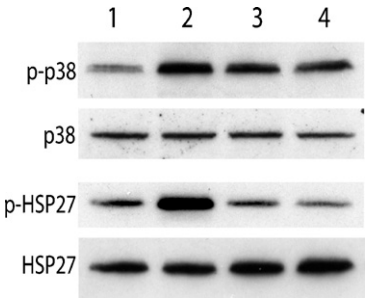


Figure 3. Effects of VX-745 treatment on the activation and activity of p38α in hTERT-immortalised WS cells. Lane 1, WS cells; lane 2, WS cells + Anisomycin (A); lane 3 WS cells + A + 1.0 μM VX-745; lane 4, WS cells + A + 2.5 μM SB203580. p-p38 and p-HSP27 indicate the phosphorylated forms of p38 and HSP27.

p-p38α, and moderate levels of p-HSP27 (lane 1). Anisomycin treatment greatly increased the activation of p38α causing an increase in p-p38α and p-HSP27 levels (lane 2). VX-745 at 1.0 μM and SB203580 at 2.5 μM inhibited the anisomycin-induced activity of p38α, as indicated by the much-reduced levels of p-HSP27 (lanes 3 and 4). The prevention of HSP27 phosphorylation appears to be due to inhibition of p38α, as VX-745 and SB203580 have at best only a minor effect on anisomycin-induced p38α activation.

In conclusion, the p38α MAPK inhibitor VX-745 can be prepared rapidly and efficiently using a combination of microwave irradiation and conductive heating methods. The four-step sequence proceeds by mono-S_NAr of 3,6-dichloropyridazine with the corresponding acetonitrile on microwave irradiation with potassium *tert*-butoxide, followed by microwave-mediated palladium-catalyzed phenylsulfide formation using PEPPSI™-IPr, hydrolysis to the amide on irradiation in concentrated sulfuric acid and treatment with *N*,*N*-dimethylformamide dimethyl acetal to facilitate the heteroannulation to the pyrimido[1,6-*b*]pyridazinone. Its inhibitory activity in HCA2 and WS cells was confirmed by ELISA and immunoblot assay, showing excellent selectivity for p38α MAPK over JNK. Given this selectivity profile, VX-745 would appear to be ideal for further studies of the accelerated ageing of WS cells in culture, which are now underway.

Acknowledgments

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References and notes

1. (a) Martin, G. M.; Oshima, J.; Gray, M. D.; Poot, M. *J. Am. Geriatr. Soc.* **1999**, *47*, 1136; (b) Yu, C.-E.; Oshima, J.; Fu, Y.-H.; Wijsman, E. M.; Hisama, F.; Alisch, R.; Matthews, S.; Nakura, J.; Miki, T.; Ouais, S.; Martin, G. M.; Mulligan, J.; Schellenberg, G. D. *Science* **1996**, *272*, 258.
2. (a) Kipling, D.; Davis, T.; Ostler, E. L.; Faragher, R. G. *Science* **2004**, *305*, 1426; (b) Kudlow, B. A.; Kennedy, B. K., ; Monnat, R. J., Jr. *Nat. Rev.* **2007**, *8*, 394.
3. Herbig, U.; Ferreira, M.; Condel, L.; Carey, D.; Sedivy, J. M. *Science* **2006**, *311*, 1257.
4. (a) Campisi, J. *Cell* **1996**, *84*, 497; (b) Faragher, R. G.; Kipling, D. *Bioessays* **1998**, *20*, 985.
5. Tollefsbol, T. O.; Cohen, H. J. *Age* **1984**, *7*, 75.
6. (a) Wright, W. E.; Shay, J. W. *Curr. Opin. Genet. Dev.* **2001**, *11*, 98; (b) Wyllie, F. S.; Jones, C. J.; Skinner, J. W.; Haughton, M. F.; Wallis, C.; Wynford-Thomas, D.; Faragher, R. G.; Kipling, D. *Nat. Genet.* **2000**, *24*, 16.
7. Baird, D. M.; Davis, T.; Rowson, J.; Jones, C. J.; Kipling, D. *Hum. Mol. Genet.* **2004**, *13*, 1515.
8. (a) Davis, T.; Baird, D. M.; Haughton, M. F.; Jones, C. J.; Kipling, D. *J. Gerontol.* **2005**, *A*, 1386; (b) Davis, T.; Wyllie, F. S.; Rokicki, M. J.; Bagley, M. C.; Kipling, D. *Ann. N.Y. Acad. Sci.* **2007**, *1100*, 455.
9. Pichierri, P.; Franchitto, A. *Bioessays* **2004**, *26*, 306.
10. Davis, T.; Kipling, D. *Rejuv. Res.* **2006**, *9*, 402.
11. Rodriguez–Lopez, A. M.; Jackson, D. A.; Iborra, F.; Cox, L. S. *Aging Cell* **2002**, *1*, 30.
12. Bagley, M. C.; Davis, T.; Dix, M. C.; Widdowson, C. S.; Kipling, D. *Org. Biomol. Chem.* **2006**, *4*, 4158.
13. (a) Dominguez, C.; Tamayo, N.; Zhang, D. *Expert Opin. Ther. Patents* **2005**, *15*, 801; (b) Goldstein, D. M.; Gabriel, T. *Curr. Top. Med. Chem.* **2005**, *5*, 1017.
14. (a) Godl, K.; Daub, H. *Cell Cycle* **2004**, *3*, 393; (b) Regan, J.; Breitfelder, S.; Cirillo, P.; Glimore, T.; Graham, A. G.; Hickey, E.; Klaus, B.; Madwed, J.; Moriak, M.; Moss, N.; Pargellis, C.; Pav, S.; Proto, A.; Swinamer, A.; Tong, L.; Torcellini, C. *J. Med. Chem.* **2002**, *45*, 2994.
15. (a) Natarajan, S. R.; Doherty, J. B. *Curr. Top. Med. Chem.* **2005**, *5*, 987; (b) Bemis, G. W.; Salituro, F. G.; Duffy, J. P.; Cochran, J. E.; Harrington, E. M.; Murcko, M. A.; Wilson, K. P.; Su, M.; Galullo, V. P. Intl. Patent WO 98/27098, 1998; (c) *Chem. Abstr.* **1998**, *129*, 81749; (d) Bemis, G. W.; Salituro, F. G.; Duffy, J. P.; Harrington, E. M. U.S. Patent 6, 147,080, 2000; (e) *Chem. Abstr.* **2000**, *133*, 350242.
16. Lee, M. R.; Dominguez, C. *Curr. Med. Chem.* **2005**, *12*, 2979.
17. Haddad, J. *Curr. Opin. Invest. Drugs* **2001**, *2*, 1070.
18. Treu, M.; Jordis, U.; Lee, V. J. *Molecules* **2001**, *6*, 959.
19. For a review, see: Ley, S. V.; Thomas, A. W. *Angew. Chem., Int. Ed.* **2003**, *42*, 5400.
20. (a) Kantchev, E. A. B.; O'Brien, C. J.; Organ, M. G. *Angew. Chem., Int. Ed.* **2007**, *46*, 2768; (b) Kantchev, E. A. B.; O'Brien, C. J.; Organ, M. G. *Aldrichim. Acta* **2006**, *39*, 97.
21. Purification by column chromatography on silica, gradient eluting with EtOAc–hexane (7:3) to EtOAc, gave VX-745 as a yellow solid, mp 261–264 °C (Found: MH^+ 435.9806, $C_{19}H_{10}N_3O^{35}Cl_2F_2S$ [MH^+] requires 435.9811); IR (nujol) ν/cm^{-1} 3019, 2399, 1626, 1600, 1215, 770, 669; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 6.50 (1H, d, *J* 9.8 Hz, 3 or 4-H), 6.80 (1H, d, *J* 9.8 Hz, 4 or 3-H), 7.00 (2H, m, 5″ and 6″-H), 7.20 (1H, t, *J* 8 Hz, 4′-H), 7.30 (2H, d, *J* 8 Hz, 3′ and 5′-H), 7.60 (1H, m, 3″-H), 8.50 (1H, s, 8-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 105.0, 113.0, 118.5, 121.5, 123.0, 125.3, 128.2, 128.4, 128.9, 129.0, 130.7, 136.5, 137.8, 149.4, 193.0; MS (ES) *m*/*z* 436 (MH^+, 100%).
22. Shi, Y.; Kotlyarov, A.; Laabeta, K.; Gruber, A. D.; Butt, E.; Marcus, K.; Meyer, H. E.; Friedrich, A.; Volk, H. D.; Gaestel, M. *Mol. Cell. Biol.* **2003**, *23*, 7732.