Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 1451–1454

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Analogues of Dealanylalahopcin Are Inhibitors of Human HIF Prolyl Hydroxylases

Imre Schlemminger,[a,†] David R. Mole,[b,†] Luke A. McNeill,[a,†] Anupma Dhanda,[a,†] Kirsty S. Hewitson,[a] Ya-Min Tian,[b] Peter J. Ratcliffe,[b] Christopher W. Pugh[b] and Christopher J. Schofield[a,*]

[a]*Oxford Centre for Molecular Sciences, Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, UK*
[b]*Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK*

Received 21 November 2002; accepted 5 February 2003

Abstract—Analogues of the naturally occurring cyclic hydroxamate dealanylalahopcin, which is an inhibitor of procollagen prolyl-4-hydroxylase, were synthesised and shown to be inhibitors of the human hypoxia-inducible factor prolyl hydroxylases.

Hypoxia-inducible factor (HIF) is a heterodimeric α,β-transcription factor present in higher organisms.[1] Under normoxic conditions, the level of the β-subunit remains constant, but levels of the α- subunit are suppressed due to proteasomal degradation. Under hypoxic conditions, levels of HIF-α rise, allowing dimerisation with HIF-β. In the nucleus, the dimer mediates activation of the array of genes involved in the hypoxic response, including those for vascular endothelial growth factor and erythropoietin.[2]

Degradation of the HIF-α subunits is triggered by post-translational hydroxylation of specific prolyl-residues that enable binding of HIF-α to the von Hippel-Lindau tumour suppressor protein (pVHL).[3,4] pVHL is part of a large ubiquitinylation complex that targets the HIF-α for transport to the proteasome and subsequent destruction.[5,6] A sub-family of the Fe(II) and 2-oxoglutarate (2-OG) dependent oxygenases (PHD1, PHD2, and PHD3 isozymes in humans) catalyses dioxygen dependent 4-hydroxylation of two prolyl residues in the oxygen dependent degradation domains of HIF-α.[7–10] Recently, hydroxylation of an asparagine residue in the C-terminal activation domain of HIF-1α has also been demonstrated;[11,12] this is proposed to mediate an hypoxic response via blocking of the interaction between HIF-α and the nuclear protein p300.

The HIF hydroxylases belong to the superfamily of 2-OG dependent non-heme iron enzymes that also contains bacterial proline hydroxylases and mammalian procollagen prolyl-4-hydroxylase (CPH).[13] Since the PHD isozymes play an integral role in oxygen homeostasis, their inhibition is attractive from the perspective of developing pharmaceuticals that induce a pro-angiogenic response for the treatment of, for example, heart disease.

In recent years several crystal structures for 2-OG dependent oxygenases have been reported.[14–17] Together with kinetic and spectroscopic studies they imply a mechanism proceeding via oxidative decarboxylation of 2-OG resulting in a (Fe(IV)=O↔Fe(III)-O·) intermediate that mediates 'prime' substrate oxidation.[18] They also reveal that in the enzyme.Fe(II).2-OG.(±substrate) complexes, the 2-OG is bound in a bidentate fashion to the iron and in an extended conformation that allows a salt bridge to form between the 5-carboxylate and a conserved arginine residue (predicted to be Arg-367 in PHD1, Arg-205 in PHD3).

Alahopcin and Analogues

Alahopcin (**1**) and dealanylalahopcin (**2**) are natural products first isolated from a sub-species of *Streptomyces*

*Corresponding author. Tel.: +44-1865-275625; fax: +44-1865-275674; e-mail: christopher.schofield@chem.ox.ac.uk
†These authors contributed equally to the work.

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00149-5

albulus.[19] Syntheses of alahopcin and dealanylalahopcin using azetidinone-2-carboxylate as a chiral template have been described.[20] In addition to the antibiotic properties of **1**, both it and **2** are reported to inhibit CPH in 2-OG decarboxylation assays.[19,21] We proposed that **2** may resemble a conformationally restrained inert analogue of 2OG, in which the hydroxamic acid coordinates the iron and the side chain carboxylate interacts with the arginine as shown in Figure 1. This proposal was investigated by the synthesis of cyclic hydroxamic acid analogues (**3**), (**4**), and (**5**), and assay of their inhibitory potential against HIF-α hydroxylation.

Thus, **3** and **4** were prepared in two steps from tricarballylic acid (**6**) and citric acid (**7**), respectively. Reflux of **6** or **7** with equimolar amounts of *O*-benzylhydroxylamine hydrochloride and pyridine in toluene under Dean-Stark conditions, yielded the cyclic *N*-hydroxysuccinimide systems (**8**) and (**9**), respectively. In the latter case a ca. 15% yield of the 6-membered ring compound (**10**) was also isolated. The *N*-benzylsuccinimides **8** and **9** were subsequently deprotected by palladium-catalyzed hydrogenation in near quantitative yield (Scheme 1).

Analogue **5** was prepared according to a literature procedure.[22] Thus, reflux of 2-methylenesuccinic acid and *O*-benzylhydroxylamine yielded the *O*-benzylated cyclic hydroxamic acid (**11**), which was quantitatively debenzylated by palladium-catalysed hydrogenation to give **5**.

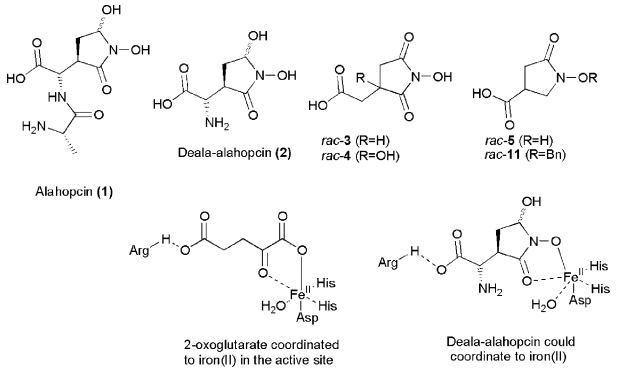
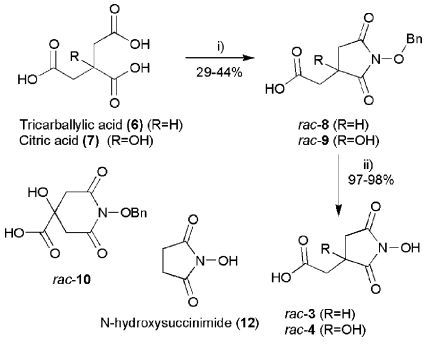


Figure 1. Dealanylalahopcin and structural related compounds, and the proposed method of iron binding by dealanylalahopcin.



Scheme 1. Synthesis of the dealanylalahopcin related compounds (**3**) and (**4**): (i) *O*-benzylhydroxylamine hydrochloride, pyridine, Dean-Stark; (ii) Pd(II)acetate, H_2.

Initially, inhibition of PHD isozymes present in mammalian cell extract (RCC4) was evaluated using a construct of HIF-1α (residues 549–582), containing the more C-terminal of the two target prolyl residues, and a Gal affinity tag that enabled immmobilisation of the peptide on agarose beads. Beads were treated with RCC4 cytoplasmic extract (made by lysing cells in 20 mM Tris pH 7.5; 5 mM KCl; 1.5 mM $MgCl_2$; 1 mM dithiothrietol at 4 °C using a Dounce homogeniser, followed by clarification at 13,000 g for 15 min) in the presence of 100 μM $FeCl_2$ both in the presence and absence of candidate inhibitors at 2 mM. The beads were then treated with ^{35}S-radiolabelled pVHL and washed extensively. The amount of radioactive pVHL captured by the hydroxylated product was analysed by denaturing PAGE electrophoresis and autoradiography (Fig. 2).

Hydroxamic acids are well-known iron-chelators in solution and at sufficient concentrations would be expected to inhibit the hydroxylation activity via simple non-selective chelation of iron. In this regard, the *O*-benzylated compounds **8**, **9** and **11** were altogether ineffective as inhibitors in the cell extract assay, probably due to absence of an *N*-hydroxyl group. The activity of the deprotected analogues containing the *N*-hydroxyl function varied significantly, suggesting that a mechanism other than, or in addition to, simple iron chelation was involved. Compound **3** was clearly a more potent inhibitor than the other compounds tested, including **5** which is a close analogue with a shorter side chain. Since **5** and *N*-hydroxysuccinimide (**12**) are expected to chelate iron as well as **3**, and exhibited no and weak inhibition of hydroxylase activity, respectively (Fig. 2 and data not shown), simple iron chelation in solution can be ruled out as a mechanism by which low levels of inhibition may be seen. It cannot be ruled out that transport phenomena play a role in the differing levels of inhibition observed in vivo. No inhibition was seen when compound **4** was tested in a similar manner using a purified fusion of maltose binding protein with PHD1 to modify a HIF-1α substrate (data not shown).

Compounds **3**, **4** and **5** were further investigated in in vitro assays using a purified fusion of maltose binding protein with PHD1 as enzyme and a 19-residue synthetic peptide containing the target Pro-564 (residues 556 – 574 from HIF-1α) as substrate. Analyses on a

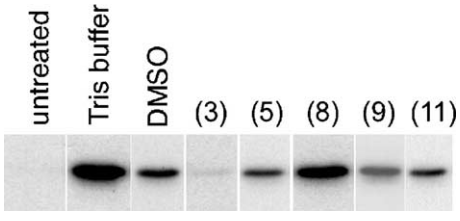


Figure 2. Autoradiographs of pVHL capture by modified HIF-α fragment immobilised on beads. Peptide not treated with mammalian cell extract is not recognised by pVHL (far left lane). Treatment of peptide with RCC4 extract and Tris buffer or DMSO shows recognition by pVHL (second and third lane), and treatment with various compounds at 2 mM varies the extent of recognition. Compounds **3** and **8** were controlled with Tris buffer, compounds **5**, **9**, and **11** with DMSO.

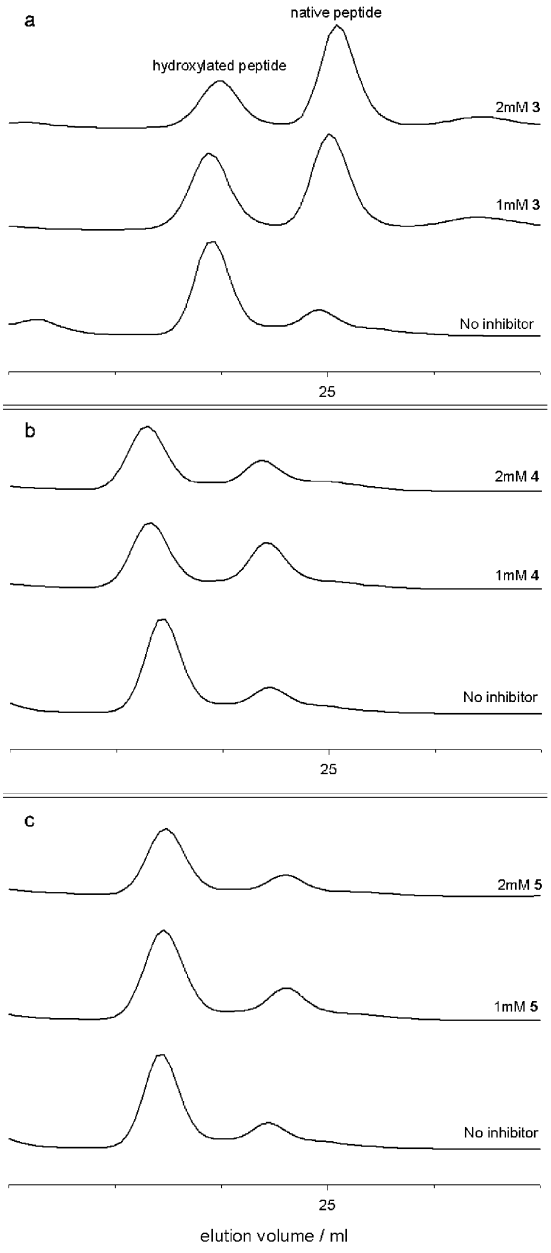
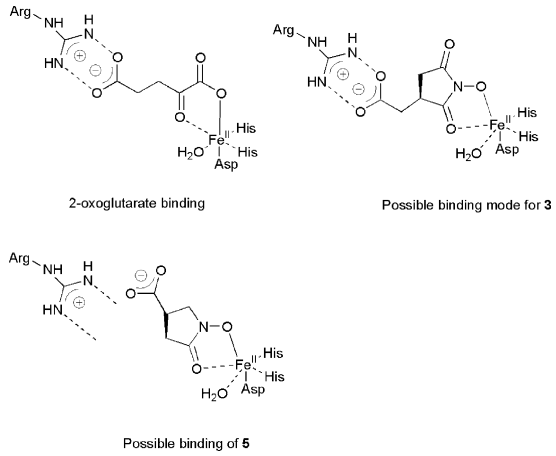


Figure 3. Comparison by HPLC, with monitoring at 214nm, of the 19mer peptide after incubation under standard conditions, except for iron(II), which was at 800 μM, with and without inhibitor. (a) with compound **3**, (b) with compound **4**, (c) with compound **5**.

reversed phase C4 hplc column (150×4.6 mm, Phenomenex) were carried out as described.[23] The results supported the in vivo results in that the actions of **3** and **5** were not solely due to simple iron chelation (Fig. 3) and gave an approximate IC_{50} value for **3** of 1mM. That the inhibition by **4** was weaker than that of **3** may suggest that the methylene carbons of 2-OG are bound by relatively tight hydrophobic interactions, as in clavaminic acid synthase[14] and taurine dioxygenase.[17] The significant difference in activity between **3** and **5** may reflect the different side chain length of the two compounds, that is, the former is a better analogue of 2-OG and bridge more effectively between the arginine and the



Scheme 2. Inferred binding of **3** and **5** to iron in the active site of the PHD isozymes. Compound **3** has the same length carbon skeleton as 2-OG but is conformationally restricted, whereas the carboxylate group of compound **5** is unsuitably positioned and cannot therefore bind so effectively.

iron in the active site (Scheme 2). Previous work has been carried out on the parallel/combnatorial synthesis of acyclic hydroxamates for screening as enzyme inhibitors.[24,25] Although the IC_{50} value of **3** for inhibition of the HIF PHDs is weak, it suggests that further structure activity work on cyclic hydroxamates may be profitable with respect to developing inhibitors of HIF prolyl hydroxylases and other metallo-enzymes.

Acknowledgements

We thank the BBSRC, EPSRC, EU, the Wellcome Trust and MRC for funding. IS is the recipient of an award from the DAAD.

References and Notes

1. Wang, G. L.; Jiang, B. H.; Rue, E. A.; Semenza, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5510.
2. Semenza, G. L. *Annu. Rev. Cell Dev. Biol.* **1999**, *15*, 551.
3. Ivan, M.; Kondo, K.; Yang, H. F.; Kim, W.; Valiando, J.; Ohh, M.; Salic, A.; Asara, J. M.; Lane, W. S.; Kaelin, W. G. *Science* **2001**, *292*, 464.
4. Jaakkola, P.; Mole, D. R.; Tian, Y. M.; Wilson, M. I.; Gielbert, J.; Gaskell, S. J.; von Kriegsheim, A.; Hebestreit, H. F.; Mukherji, M.; Schofield, C. J.; Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. J. *Science* **2001**, *292*, 468.
5. Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. *J. Exp. Nephrol.* **2001**, *9*, 235.
6. Cockman, M. E.; Masson, N.; Mole, D. R.; Jaakkola, P.; Chang, G. W.; Clifford, S. C.; Maher, E. R.; Pugh, C. W.; Ratcliffe, P. J.; Maxwell, P. H. *J. Biol. Chem.* **2000**, *275*, 25733.
7. Yu, F.; White, S. B.; Zhao, Q.; Lee, F. S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9630.
8. Masson, N.; Willam, C.; Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. J. *EMBO J.* **2001**, *20*, 5197.
9. Epstein, A. C. R.; Gleadle, J. M.; McNeill, L. A.; Hewitson, K. S.; O'Rourke, J.; Mole, D. R.; Mukherji, M.; Metzen, E.; Wilson, M. I.; Dhanda, A.; Tian, Y. M.; Masson, N.; Hamilton, D. L.; Jaakkola, P.; Barstead, R.; Hodgkin, J.; Maxwell, P. H.; Pugh, C. W.; Schofield, C. J.; Ratcliffe, P. J. *Cell* **2001**, *107*, 43.

10. Bruick, R. K.; McKnight, S. L. *Science* **2001**, *294*, 1337.
11. Lando, D.; Peet, D. J.; Gorman, J. J.; Whelan, D. A.; Whitelaw, M. L.; Bruick, R. K. *Genes Dev*. **2002**, *16*, 1466.
12. Hewitson, K. S.; McNeill, L. A.; Riordan, M. V.; Tian, Y. M.; Bullock, A. N.; Welford, R. W. D.; Elkins, J. M.; Oldham, N. J.; Battacharya, S.; Gleadle, J.; Ratcliffe, P. J.; Pugh, C. W.; Schofield, C. J. *J. Biol. Chem*. **2002**, *277*, 26351.
13. Aravind, L.; Koonin, E. V. *Genome Biology* **2001**, *2*, research0007.1.
14. Zhang, Z.; Ren, J.; Stammers, D. K.; Baldwin, J. E.; Harlos, K.; Schofield, C. J. *Nat. Struct. Biol*. **2000**, *7*, 127.
15. Clifton, I. J.; Hsueh, L. C.; Baldwin, J. E.; Harlos, K.; Schofield, C. J. *Eur. J. Biochem*. **2001**, *268*, 6625.
16. Valegard, K.; van Scheltinga, A. C. T.; Lloyd, M. D.; Hara, T.; Ramaswamy, S.; Perrakis, A.; Thompson, A.; Lee, H. J.; Baldwin, J. E.; Schofield, C. J.; Hajdu, J.; Andersson, I. *Nature* **1998**, *394*, 805.
17. Elkins, J. M.; Ryle, M. J.; Clifton, I. J.; Dunning Hotopp, J. C.; Lloyd, J. S.; Burzlaff, N. I.; Baldwin, J. E.; Hausinger, R. P.; Roach, P. L. *Biochemistry* **2002**, *41*, 5185.
18. Lloyd, M. D.; Lee, H. J.; Harlos, K.; Zhang, Z. H.; Baldwin, J. E.; Schofield, C. J.; Charnock, J. M.; Garner, C. D.; Hara, T.; van Scheltinga, A. C. T.; Valegard, K.; Viklund, J. A. C.; Hajdu, J.; Andersson, I.; Danielsson, A.; Bhikhabhai, R. *J. Mol. Biol*. **1999**, *287*, 943.
19. Higashide, E.; Horii, S.; Ono, H.; Mizokami, N.; Yamazaki, T.; Shibata, M.; Yoneda, M. *J. Antibiot*. **1985**, *38*, 285.
20. Baldwin, J. E.; Adlington, R. M.; Godfrey, C. R. A.; Gollins, D. W.; Schofield, C. J. *Tetrahedron* **1991**, *47*, 5835.
21. Higashide, E.; Kanamaru, T.; Fukase, H.; Horii, S. *J. Antibiot*. **1985**, *38*, 296.
22. Akiyama, M.; Shimizu, K.; Aiba, S.; Katoh, H. *Bull. Chem. Soc. Jpn*. **1985**, *58*, 1421.
23. McNeill, L. A.; Hewitson, K. S.; Gleadle, J.; Horsfall, L. E.; Oldham, N. J.; Maxwell, P.; Pugh, C. W.; Ratcliffe, P. J.; Schofield, C. *J. Bioorg. Med. Chem. Lett*. **2002**, *12*, 1547.
24. Volonterio, A.; Bellosta, S.; Bravo, P.; Canavesi, M.; Corradi, E.; Meille, S.V.; Monetti, M.; Moussier, N.; Zanda, M. *Eur. J. Org. Chem*. **2002**, *3*, 428.
25. Szardenings, A. K.; Harris, D.; Lam, S.; Shi, L. H.; Tien, D.; Wang, Y. W.; Patel, D. V.; Navre, M.; Campbell, D. A. *J. Med. Chem*. **1998**, *41*, 2194.