Tetrahedron Letters 50 (2009) 3151–3153

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Evaluation of chlorofusin, its seven chromophore diastereomers, and key analogues

Ryan C. Clark [a], Sang Yeul Lee [a], Inkyu Hwang [a], Mark Searcey [b], Dale L. Boger [a,*]

[a] Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA
[b] School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, NR4 7TJ, UK

ARTICLE INFO

Article history:
Received 28 December 2008
Accepted 15 January 2009
Available online 20 January 2009

ABSTRACT

Chlorofusin, its seven chromophore diastereomers, and key analogues were comparatively examined for inhibition of MDM2–p53 binding revealing that the chromophore, but not simple replacements, contributes significantly to the natural products properties, and that this contribution is independent of its relative and absolute stereochemistry.

The tumor suppressor p53 is an important part of an innate cancer defense mechanism and acts as a transcription factor that initiates cell cycle arrest and apoptosis in response to stress such as DNA damage.[1–4] The activity of p53 is modulated by MDM2 (HDM2), which tightly binds p53 preventing it from acting as a regulator of cell division[5–7] and targeting it for nuclear export and degradation.[8,9] Overexpression of MDM2 has been implicated in many cancers, defining the p53–MDM2 interaction as an attractive target for therapeutic intervention.[4] An X-ray crystal structure of the N-terminal domain of MDM2 bound to a 15-residue transactivation domain of p53 revealed the structural details of their complex that is mediated by the interaction of three hydrophobic residues of a p53 α-helix with a hydrophobic cleft of MDM2.[10] Molecules that bind the hydrophobic cleft of MDM2 disrupt this protein–protein interaction with p53, restoring its regulatory function and inhibiting tumor growth.[4,11]

Chlorofusin (**1**, Fig. 1) was isolated from the fungal strain *Microdochium caespitosum*[12] and reported to disrupt the MDM2–p53 interaction by directly binding to the N-terminal domain of MDM2 (IC_{50} = 4.6 μM, K_D = 4.7 μM).[12–14] Thus, chlorofusin has been regarded as an exciting lead for antineoplastic intervention that acts by a rare disruption of a protein–protein interaction, although the structural details of this inhibitory interaction derived from MDM2 binding have yet to be established.[15] On the basis of extensive spectroscopic and degradation studies, the chlorofusin structure was proposed to consist of a densely functionalized, azaphilone-derived chromophore linked through the terminal amine of ornithine to a 27-membered cyclic peptide composed of nine amino acid residues.[12]

Although the studies permitted the identification of the cyclic peptide structure and connectivity, the two asparagine residues Asn3 and Asn4 were only established to have opposite stereochemistries (L and D) and their respective assignments were not possible. Similarly, the spectroscopic studies conducted by Williams provided the structure and an assigned relative stereochemistry for the unusual azaphilone-derived chromophore, but did not permit an assignment of its absolute stereochemistry. In early studies, we reported the synthesis of the two cyclic peptide diastereomers bearing either the L-Asn3/D-Asn4 or D-Asn3/L-Asn4 stereochemistry and correlation of the former with the spectroscopic properties (^{1}H and ^{13}C NMR) of the natural product.[16] Concurrent with this disclosure, Searcey reported the synthesis of the L-Asn3/D-Asn4 diastereomers incorporating either a D-ADA8 or L-ADA8 residue confirming its stereochemical assignment,[17] and recently Nakata[18] has reported a synthesis of this cyclic peptide.

Most recently, we reported studies leading to a reassignment of the relative stereochemistry for the chlorofusin chromophore as well as to the assignment of the chromophore absolute configuration (4*R*,8*S*,9*R*).[19] In this work and in addition to natural

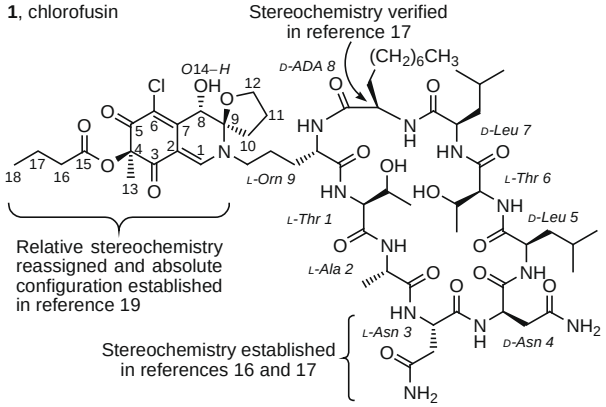


Figure 1. Structure of chlorofusin.

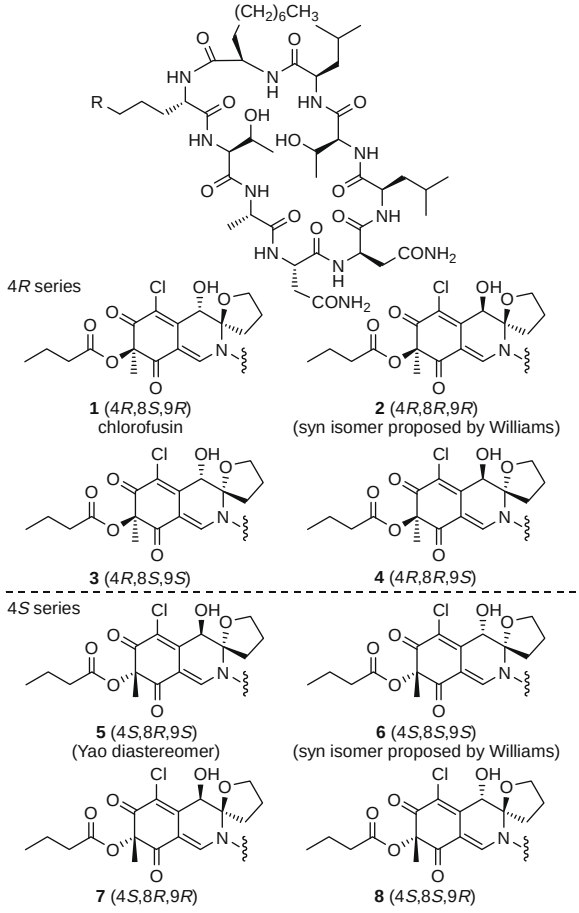
* Corresponding author. Tel.: +1 858 784 7522; fax: +1 858 784 7550.
E-mail address: boger@scripps.edu (D.L. Boger).

0040-4039/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2009.01.081

chlorofusin, the remaining seven chlorofusin chromophore diastereomers including the (4*R*,8*R*,9*R*)/(4*S*,8*S*,9*S*)-diastereomers proposed by Williams[12] as well as a diastereomer misinterpreted as the natural product by Yao[20] (4*S*,8*R*,9*S*) were synthesized as key analogues of the natural product and for comparison of their spectral properties with that reported for chlorofusin. In addition to providing unambiguous support for the structural reassignment, this provided the opportunity to explore and define key structure–activity relationships (SAR) for the inhibition of p53–MDM2 (p53–HDM2) binding. Notably, there are no apparent structural relationships between the residues found in the chlorofusin cyclic peptide and the p53 α-helix mediating the binding to the hydrophobic cleft of MDM2[17] and the nature of chlorofusin interaction with the N-terminal domain of MDM2 established by surface plasma resonance (SPR)[13] remains to be defined. Herein, we report the assessment of **1**–**8** and several additional key analogues and partial structures of chlorofusin for inhibition of p53–MDM2 binding.

The assay adopted to examine chlorofusin and its analogues was an ELISA competition assay described by Searcey.[21] In this assay, the analogues are examined for their ability to disrupt the binding of MDM2 (available from EMD Biosciences) and a biotinylated SGSG-p53 peptide (17–27) that is immobilized on streptavidin-coated 96-well plates. A control p53 peptide (SGETFSDLWKLLPEN) as well as nutlin-1, a well-established standard, produced IC_{50}'s comparable to those reported (4.1 μM and 0.13 μM, respectively). Significant in the selection of this assay is the complete lack of any detectable background signal in the absence of immobilized p53 peptide. This permitted reproducible detection and accurate quantitation of inhibited binding by weak inhibitors without the complication of a significant background signal. Notably and over the course of several years, we examined multiple assays for monitoring p53–MDM2 binding and the Searcey assay proved by far to be the most reliable. Additionally, we were not able to convince potential collaborators to adopt the original DELFIA modified ELISA used to identify **1**[12] and we were unable to secure the necessary proteins needed to reproduce the assay ourselves. Attempts to use more contemporary assays that enlist fluorescent quantitation failed, since the intrinsic fluorescence of chlorofusin was found to interfere with the binding measurements. Using the Searcey assay, chlorofusin and each of its seven chromophore diastereomers proved to be effective inhibitors of p53–MDM2 binding (Fig. 2). Chlorofusin was the most effective of the compounds evaluated displaying a potency that is comparable to that reported (IC_{50} = 8 μM), but all exhibited activity within threefold of the natural product. Three additional chromophore diastereomers (**3**, **5**, and **8**) were equipotent with chlorofusin including two that are in the unnatural 4*S* series. Most notable of these is **5** in which the chromophore is enantiomeric with that of the natural product. These surprising results, in which the chromophore relative and absolute stereochemistry play little or no role, are especially interesting given that the simplified derivatives **9**–**12** of the cyclic peptide, in which the chromophore was simply removed (**9**, R = NH_2) or replaced with large hydrophobic amine protecting groups, proved to be >10-fold less potent displaying IC_{50}'s > 100 μM. The inactivity of such derivatives of the chlorofusin cyclic peptide has been disclosed previously and was confirmed herein.[16,17,21] The relative inactivity of **10**, bearing a Cbz group in place of the chromophore, is noteworthy given that it may be viewed as an achiral unfunctionalized replacement.

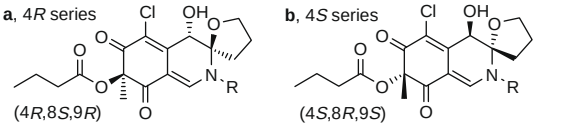
Just as significant, the entire series of partial structures that contain each chromophore diastereomer bearing a *N*-butyl, *N*-benzyl, and N^{ε}-linked FmocHN-Thr-Orn-OBn or H_2N-Thr-Orn-OBn all displayed IC_{50}'s > 100 μM and each was >10-fold less active than chlorofusin (Fig. 2). This is illustrated in Figure 2 with the



Inhibition of p53–MDM2 binding, IC_{50} (μM)

Isomer	IC_{50}	Isomer	IC_{50}
1, chlorofusin	8 μM[a]	**5**	8 μM
2	10 μM[b]	**6**	18 μM[c]
3	8 μM	**7**	16 μM[c]
4	24 μM	**8**	8 μM
9, R = NH_2	>100 μM	**11**, R = NHBoc	150 μM[d]
10, R = NHCbz	140 μM[c]	**12**, R = NHSES	150 μM[d]

The results are the average of [a]10, [b]2, [c]3, or [d]5 determinations run in triplicate.



Inhibition of p53–MDM2 binding, IC_{50} (μM)

Compound	IC_{50}	Compound	IC_{50}
13a, R = Bu	250 μM	**13b**, R = Bu	170 μM
14a, R = Bn	>100 μM	**14b**, R = Bn	>100 μM
15a, R = A	150 μM	**15b**, R = A	170 μM
16a, R = B	150 μM	**16b**, R = B	150 μM

Figure 2. Evaluation of chlorofusin and key analogues.

(4*R*,8*S*,9*R*)- and (4*S*,8*R*,9*S*)-diastereomers, but is representative of all 32 compounds examined. Like the observations made with **1**–**8**, no distinctions or discernable trends were observed that reflect the relative or absolute stereochemistry of the chromophore.

Finally, concerned that this behavior of **1**–**8** may simply represent promiscuous inhibition of the protein–protein interaction by chlorofusin aggregates, the assay was conducted with chloro-

fusin and nutlin-1 in the presence of 0.01% Triton.[22] This had no effect on the measured IC_{50}'s, suggesting that **1** and the related analogues **2–8** are not acting as promiscuous aggregate inhibitors.

As such, the results provide a clear, but unusual depiction of the structural features of **1** required for inhibition of p53–MDM2 binding. Neither the chlorofusin cyclic peptide (**9**) and its simple derivatives (e.g., **10–12**) nor simple derivatives of the chlorofusin chromophore (e.g., **13–16**) exhibit significant inhibition of this protein–protein interaction. In contrast, chlorofusin and each of its seven chromophore diastereomers exhibit effective, albeit not especially potent, inhibition of p53–MDM2 binding that was essentially independent of the chromophore relative and absolute stereochemistry. Our present studies are not able to distinguish whether this reflects the impact of a flexible linker joining the two domains of the natural product or whether it may reflect an as yet undetected reversible, covalent attachment of the chromophore driven by weak cyclic peptide binding.[23]

Acknowledgments

We gratefully acknowledge the financial support of the National Institutes of Health (CA41101) and the Skaggs Institute for Chemical Biology. RCC and SYL are Skaggs Fellows.

References and notes

1. Levine, A. J.; Momand, J.; Finlay, C. A. *Nature* **1991**, *351*, 453.
2. Vousden, K. H. *Cell* **2000**, *103*, 691.
3. Shen, Y.; White, E. *Adv. Cancer Res.* **2001**, *82*, 55.
4. Chéne, P. *Nat. Rev. Cancer* **2003**, *3*, 102; Zhang, R.; Wang, H. *Curr. Pharm. Des.* **2000**, *6*, 393.
5. Momand, J.; Zambetti, G. P.; Olson, D. C.; George, D.; Levine, A. J. *Cell* **1992**, *69*, 1237.
6. Chen, J.; Marechal, V.; Levine, A. J. *Mol. Cell. Biol.* **1993**, *13*, 4107.
7. Oliner, J. D.; Pietenpol, J. A.; Thiagalingam, S.; Gyuris, J.; Kinzler, K. W.; Vogelstein, B. *Nature* **1993**, *362*, 857.
8. Haupt, Y.; Maya, R.; Kazaz, A.; Oren, M. *Nature* **1997**, *387*, 296.
9. Kubbutat, M. H. G.; Jones, S. N.; Vousden, K. H. *Nature* **1997**, *387*, 299.
10. Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. *Science* **1996**, *274*, 948.
11. Bullock, A. N.; Fersht, A. R. *Nat. Rev. Cancer* **2001**, *1*, 68; Fischer, P. M.; Lane, D. P. *Trends Pharmacol. Sci.* **2004**, *25*, 343.
12. Duncan, S. J.; Grueschow, S.; Williams, D. H.; McNicholas, C.; Purewal, R.; Hajek, M.; Gerlitz, M.; Martin, S.; Wrigley, S. K.; Moore, M. *J. Am. Chem. Soc.* **2001**, *123*, 554; Correction: . *J. Am. Chem. Soc.* **2002**, *124*, 14503.
13. Duncan, S. J.; Cooper, M. A.; Williams, D. H. *Chem. Commun.* **2003**, 316.
14. Clark, R.C.; Lee, S.Y.; Searcey, M.; Boger, D.L. *Nat. Prod. Rep.* **2009**, *26*, in press.
15. Boger, D. L.; Desharnais, J.; Capps, K. *Angew. Chem., Int. Ed.* **2003**, *42*, 4138.
16. Desai, P.; Pfeiffer, S. S.; Boger, D. L. *Org. Lett.* **2003**, *5*, 5047; Lee, S. Y.; Boger, D. L. *Tetrahedron* **2008**, *49*, 6585.
17. Malkinson, J. P.; Zloh, M.; Kadom, M.; Errington, R.; Smith, P. J.; Searcey, M. *Org. Lett.* **2003**, *5*, 5051.
18. Mori, T.; Miyagi, M.; Suzuki, K.; Shibasaki, M.; Saikawa, Y.; Nakata, M. *Heterocycles* **2007**, *72*, 275.
19. Lee, S. Y.; Clark, R. C.; Boger, D. L. *J. Am. Chem. Soc.* **2007**, *129*, 9860; Clark, R. C.; Lee, S. Y.; Boger, D. L. *J. Am. Chem. Soc.* **2008**, *130*, 12355.
20. Qian, W.-J.; Wei, W.-G.; Zhang, Y.-X.; Yao, Z.-J. *J. Am. Chem. Soc.* **2007**, *129*, 6400.
21. Woon, E. C. Y.; Arcieri, M.; Wilderspin, A. F.; Malkinson, J. P.; Searcey, M. *J. Org. Chem.* **2007**, *72*, 5146.
22. McGovern, S. L.; Helfand, B. T.; Feng, B.; Shoichet, B. K. *J. Med. Chem.* **2003**, *46*, 4265; Seidler, J.; McGovern, S. L.; Doman, T. N.; Shoichet, B. K. *J. Med. Chem.* **2003**, *46*, 4477.
23. p53/HDM2 Inhibition Assay. A streptavidin-coated 96-microwell plate (ReactiBind™ Streptavidin High Binding Capacity-coated-plate, Pierce) was rinsed with wash buffer (PBS + 0.05% Tween-20, 3×) and incubated with 100 μL per well of biotinylated SGSG-p53 peptide (1727, 50 nM in wash buffer) at 4 °C for 1 h. Any unbound peptide was then removed by rinsing with wash buffer (3×). In a separate 96-microwell polypropylene plate (Pierce), 50 μL of HDM2 (4 nM, EMD Bioscience) was incubated with 50 μL of inhibitor (a range of concentrations) at 4 °C for 30 min in a Wallac assay buffer (Perkin Elmer, Product No. 1244–111). The HDM2/inhibitor mixtures (100 μL) were then transferred to a well in the streptavidin-coated plate and were left to incubate at 4 °C for 1 h. The wells were then washed with wash buffer (5×), followed by the addition of 100 μL of anti-HDM2 [(Ab-6) mouse mAb(5B10C)] (EMD Bioscience), diluted 500-fold with a dilution buffer (PBS + 0.05% Tween-20 + 1% bovine serum albumin). After 2 h at 4 °C, the wells were washed with wash buffer (5×) and a solution of anti-mouse IgG conjugated with horseradish peroxidase (Jackson Immunochemical), diluted in dilution buffer (1 μg/mL), was added. The wells were incubated at 4 °C for 1 h before being washed with wash buffer (5×). A 100 μL solution of peroxidase substrate, 3,3′,5′,5-tetramethylbenzidine (Sigma–Aldrich), was added and the reaction was allowed to incubate at room temperature for 30 min to develop a blue product. Quenching the reaction with 100 μL of aqueous HCl (0.15 N) produces a yellow color which can be read at 450 nm. The percentage of p53/HDM2 binding was calculated, from which the IC_{50} values for the analogues were determined. The assay was performed in triplicate using blank, which does not contain SGSG-p53 peptide as a negative control.