Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 507–510
Bioorganic & Medicinal Chemistry Letters

Synthesis and activity of a potent, specific azabicyclo[3.3.0]-octane-based DPP II inhibitor

Olga Danilova,[a] Bei Li,[b] A. Katrin Szardenings,[b] Brigitte T. Huber[a,*] and Jonathan S. Rosenblum[b,*]

[a]*Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111, USA*
[b]*ActivX Biosciences, Inc., 11025 North Torrey Pines Road, La Jolla, CA 92037, USA*

Received 13 September 2006; revised 5 October 2006; accepted 5 October 2006
Available online 10 October 2006

Abstract—A cell permeable DPP II [also known as DPP2, DPP7, and quiescent cell proline dipeptidase (QPP)] inhibitor has been synthesized. The azabicyclo[3.3.0]octane-based inhibitor is potent and selective and elicits very similar quiescent lymphocyte death to previously characterized inhibitors that are not as selective.

Recently, a family of serine proteases with the common ability to cleave after prolines has attracted attention as potential drug targets.[1,2] The most widely studied enzyme in this family is dipeptidyl peptidase (DPP) IV (also known as the T-cell antigen CD26), which is a clinically validated target for type 2 diabetes.[3] DPP IV is responsible for the rapid inactivation of the incretin GLP-1 by virtue of its ability to remove an amino-terminal dipeptide.[4] It is possible that other members of the family function in analogous fashions, but determining the precise function of the other members has lagged for a number of reasons, including the unavailability of genetically modified animals, paucity of known biological substrates for the proteases, and absence of specific, biologically active inhibitors.

In addition to DPP IV, the members include fibroblast activation protein α (FAP), DPP II (also known as DPP2, DPP7, and quiescent cell proline dipeptidase or QPP), prolylcarboxypeptidase, DPP8, DPP9, acylpeptide hydrolase (APH), and prolyl oligopeptidase (POP). These enzymes share the ability to cleave peptides with proline (and sometimes alanine) at the P1-position, and some of the enzymes have specificity for P2 amino acids as well.[5,6] In addition to demonstrating different levels of expression and tissue distribution, members of this family are found extracellularly (DPP IV), bound to the plasma membrane (DPP IV and FAPα), in the cytoplasm (DPP8, DPP9, POP, and APH), and in specialized vesicles (DPP II).[7–12]

DPP II, a 58 kDa glycoprotein, is localized to intracellular vesicles distinct from lysozymes and can be secreted in active form in response to calcium release.[13] Homodimerization via a leucine zipper motif is required for DPP II catalytic activity.[14] It is active within a broad range of pH with optimum between 5.5 and 7.0.[5,8] It is believed that DPP II is essential for the G_0 survival program of lymphocytes and neuronal cells. Inhibition of DPP II induces apoptosis of these quiescent cells.[15] DPP II may also be involved in pathogenesis of B cell chronic lymphocytic leukemia (B-CLL). B cells arrested in G_0 accumulate in peripheral blood of CLL patients. Susceptibility to DPP II-induced apoptosis serves as a prognostic factor of CLL outcome.[16] Natural DPP II substrates remain unknown.

In order to gain further understanding of the biological role(s) of DPP II, we and others have synthesized small molecule inhibitors.[17–19] A common starting point for the synthesis of DPP II inhibitors is the cationic P2 preference of the enzyme.[5] For example, 2,4-diaminobutanoic acid (Dab) has been used as a P2 group in dipeptide inhibitors, where P1 was piperidine or boronorleucine. Inhibitors of this type are typically potent and highly selective for DPP II over the other DPP enzymes (see Fig. 1 for representative DPP II inhibitors).

Keyword: Dipeptidyl peptidase.
* Corresponding authors. Tel.: +1 617 636 3989; fax: +1 617 636 2990 (B.T.H); tel.: +1 858 558 5558; fax: +1 858 587 4878 (J.S.R.); e-mail: jonr@activx.com

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.10.012

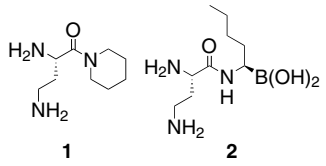

Figure 1. Representative DPP II inhibitors.[18,17]

However, such hydrophilic inhibitors may suffer from low cell permeability, which could render them unable to target the intracellular compartment, where DPP II is found.

Here, we report the discovery and biological characterization of a potent and selective azabicyclo[3.3.0]octane-based DPP II inhibitor.

In the course of exploring potential P1 groups for DPP II inhibitors, we focused on structures that could take advantage of DPP II's larger S1 site relative to DPP IV, with a preference for lipophilic structures that would counterbalance the polar Dab group. Of the groups that were explored, the azabicyclo[3.3.0]octane shown in Figure 2 demonstrated the best combination of potency and specificity for DPP II.

AX8819 was prepared by a standard coupling of amine **3**[20] with Boc-*N*-2,4-diaminobutyric acid followed by HCl deprotection of the Boc groups.

AX8819 was tested for potency against members of the DPP family in cell-free extracts. As shown in Table 1, AX8819 is potent and selective for DPP II. Other compounds used in these studies were assayed side-by-side with AX8819 and their IC_{50} values are also shown in Table 1. We next tested the ability of the compounds to inhibit intracellular DPP II when added to intact cells. Testing the inhibitors in intact cells allows the determination of several factors, including cell permeability of the compound, the ability to target the DPP II-containing vesicles, compound potency, and stability.

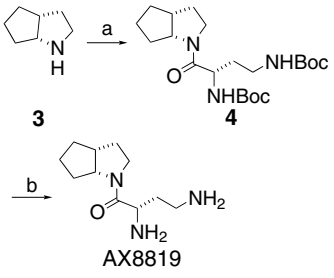

Figure 2. Synthesis of AX8819. (a) Boc-*N*-2,4-diaminobutyric acid, DIEA, HOBt, EDC, DMF. (b) 4 M HCl in dioxane, rt, 1 h.

Table 2. Remaining Ala-Pro-AFC cleavage activity (%) upon treating intact cells with compounds[21]

Compound (concentration)	DPP II-293T HEK	293T HEK	Jurkat
VbP (10 μM)	75.3	3.9	43.7
2 (4.8 μM)	62.4	94.3	
AX8819 (0.88 μM)	65.48	97.3	
AX8819 (4.4 μM)	60.6	115.5	75.6
AX8819 (8.8 μM)	57.7	65.8	68.4
AX8819 (22 μM)	21.1	91.1	52.4
AX8819 (44 μM)	28.9	96.1	46.8

Three DPP II inhibitors were tested (Tables 1 and 2). The original cell-based potency testing was performed using DPP II-293T HEK and 293T HEK cell lines. DPP II-293T HEK, a cell line that overexpresses human DPP II,[14] was used to test the compounds' permeability and selectivity. The 293T HEK cell line was employed as a control, since it does not express DPP II, but has other DPP activities (DPP8 and 9) and can be used to test the specificity of the compound. We used ValBoroPro (VbP[1]) as a standard non-specific DPP inhibitor (Table 1). As seen in Table 2, the compounds required between 1000 and 10,000 times their IC_{50} concentrations to inhibit approximately half of the DPP activity in DPP II overexpressing HEK cells (DPP II-293 T HEK column). As mentioned above, this level of compound reflects a combination of cell- and organelle-permeability, compound stability, and other factors.[22] Taken together these results indicate that compounds **2** and AX8819 appear to bracket the intact cell DPP II inhibition of VbP, while being substantially more selective for DPP II over all of the other DPP enzymes.

Compounds **2** and AX8819 were further studied for general toxicity. As observed previously, inhibition of DPP II does not cause cell death of proliferating Jurkat cells.[15] Hence, if cell death is observed it is likely due to the toxicity of the studied compound. None of the compounds killed more than 5% of treated Jurkat cells at 5000-fold their DPP II IC_{50} after 16 h of incubation. AX8819 did not cause any cell death in Jurkat cells at concentrations up to 44 μM, 50,000-fold its DPP II IC_{50}. By this assay AX8819 was therefore judged to have very low non-specific toxicity. VbP and **2** also exhibit low non-specific toxicity toward Jurkat cells.

We continued to examine functionality and specificity of AX8819 and to explore the consequences of DPP II inhibition on a cellular level. Also our goal was to deter-

Table 1. IC_{50} values of compounds tested in cell-free extracts[a]

Compound	DPP IV IC_{50} (nM)	DPP II IC_{50} (nM)	FAP IC_{50} (nM)	DPP8 IC_{50} (nM)	DPP9 IC_{50} (nM)	POP IC_{50} (nM)
AX8819	>33,000	0.88	>33,000	>20,000	>20,000	>20,000
2	>33,000	0.48	>33,000	4300	800	>20,000
VbP	0.10	30	24	17	2.0	7960

[a] IC_{50} values were determined as described previously.[17,21]

mine the most efficient concentration of the inhibitor to use in intact cell applications. A range of doses of AX8819 were used to explore its ability to selectively inhibit DPP II when added to intact cells. AX8819 significantly inhibited the overexpressed DPP II in DPP II-293T HEK cells at all used concentrations and the endogenous DPP II in Jurkat cells at 22 and 44 μM doses. At the same time, at up to 44 μM, it did not significantly inhibit the DPP activity of 293T HEK cells (Table 2). Thus, AX8819 demonstrated characteristics of a potent and specific inhibitor at a range of doses. At concentrations of up to 44 μM, AX8819 did not kill proliferating Jurkat cells or inhibit other DPP enzymes in untransfected 293 cells.

As mentioned above, DPP II is involved in survival of resting cells. Inhibition of DPP II in these cells causes apoptosis. A series of cell death assays were performed on resting freshly isolated human peripheral blood mononuclear cells (PBMCs) to demonstrate the results of specific DPP II inhibition. Apoptotic cell death was analyzed by annexin V (AnV) and propidium iodide (PI) staining.[23]

Resting PBMCs were treated with various doses of AX8819 and 10 μM VbP for 4, 8, and 16 h. Cells were washed and stained with AnV-allophycocyanin, and propidium iodide was added immediately prior to analysis on FACScalibur. AX8819 caused cell death at 22 and 44 μM. The kinetics and extent of cell death caused by 44 μM AX8819 were identical to those of 10 μM VbP, while 22 μM AX8819 led to slightly less cell death and concentrations of AX8819 below 10 μM were indistinguishable from DMSO (Fig. 3). AX8819 caused apoptotic cell death (4-fold increase in the number AnV^+/PI^- and AnV^+/PI^+ cells) of resting PBMC comparable to VbP.[24,25] The difference in the number of AnV^+ cells between AX8819 (22 and 44 μM) and VbP (10 μM) treated cells compared to DMSO control is significant (*t* test, $p < 0.001$) at the 16 h time point.

To confirm the apoptotic nature of cell death, cells were treated with 100 μM zVAD-fmk 1 h prior to compound

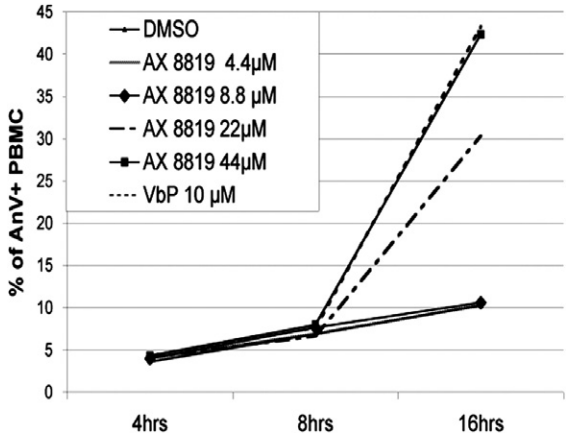

Figure 3. Kinetics of apoptotic death of PBMC upon DPP II inhibition.

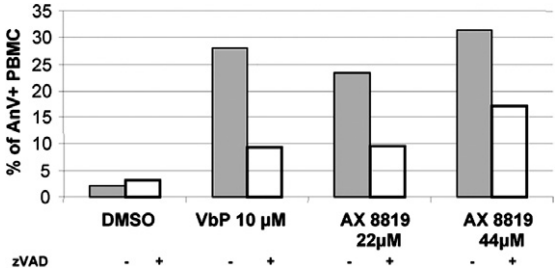

Figure 4. Pretreatment of resting PBMC with 100 μM zVAD-fmk reduces compound dependent cell death.

addition. For each compound, cell death was compared in the presence and absence of active caspases (with and without zVAD-fmk, Fig. 4). A significant reduction (paired *t* test, $p < 0.05$) in the numbers of AnV^+ cells was observed in the samples that were pre-treated with zVAD-fmk and incubated with AX8819 and VbP, thus confirming that cell death caused by DPP II inhibition is caspase-dependent.

A recent report[26] described studies with a DPP II inhibitor that apparently did not cause any form of cell death when PBMCs were treated with a cell permeable DPP II inhibitor. No cell death was seen with the DPP II inhibitor, but cells were killed with etoposide, a topoisomerase II inhibitor used in cancer chemotherapy. Etoposide triggers apoptosis through a mechanism distinct from that initiated by the DPP II inhibitors. For example, etoposide causes apoptosis in Jurkat cells[27] and DPP II inhibitors do not. Unfortunately, since Maes et al. did not use a DPP II inhibitor shown previously to cause apoptosis in PBMC, for example VbP, as a positive control, it is possible that their PBMCs are resistant to apoptosis via this mechanism. It is important to note that we have observed that PBMCs derived from different individuals exhibit various degrees of susceptibility to DPPII inhibition-induced apoptosis (unpublished observation). Since some non-cycling cells that have DPP II activity are not susceptible to DPP II inhibition-induced apoptosis, it is clear that other factors are required to explain this phenotype.

In conclusion, AX8819 is a novel DPP II-specific inhibitor. Its functionality, specificity, and potency were demonstrated in several cell-based systems. Though uniquely specific to DPP II among the DPP enzymes, AX8819 is able to kill PBMC in a similar fashion to VbP, a pan DPP inhibitor. As such, AX8819 further supports our hypothesis that DPP II is the relevant target in the apoptosis of quiescent cells and could be a useful tool in further elucidation of the biological role of DPP II.

Acknowledgments

We thank John Kozarich for guidance and insightful suggestions and Kevin Shreder for supplying compound **2**. This work was supported by the NIH (RO1 AI 43469; B.T.H.) and GRASP Center P30 DK 34928 (B.T.H.).

References and notes

1. Rosenblum, J. S.; Kozarich, J. W. *Curr. Opin. Chem. Biol.* **2003**, *7*, 496.
2. Augustyns, K.; Van der Veken, P.; Senten, K.; Haemers, A. *Curr. Med. Chem.* **2005**, *12*, 971.
3. Ahren, B. *Expert Opin. Investig. Drugs* **2006**, *15*, 431.
4. Deacon, C. F. *Regul. Pept.* **2005**, *128*, 117.
5. Leiting, B.; Pryor, K. D.; Wu, J. K.; Marsilio, F.; Patel, R. A.; Craik, C. S.; Ellman, J. A.; Cummings, R. T.; Thornberry, N. A. *Biochem. J.* **2003**, *371*, 525.
6. Edosada, C. Y.; Quan, C.; Wiesmann, C.; Tran, T.; Sutherlin, D.; Reynolds, M.; Elliott, J. M.; Raab, H.; Fairbrother, W.; Wolf, B. B. *J. Biol. Chem.* **2006**, *281*, 7437.
7. Sun, S.; Albright, C. F.; Fish, B. H.; George, H. J.; Selling, B. H.; Hollis, G. F.; Wynn, R. *Protein Expr. Purif.* **2002**, *24*, 274.
8. Underwood, R.; Chiravuri, M.; Lee, H.; Schmitz, T.; Kabcenell, A. K.; Yardley, K.; Huber, B. T. *J. Biol. Chem.* **1999**, *274*, 34053.
9. Shariat-Madar, Z.; Mahdi, F.; Schmaier, A. H. *J. Biol. Chem.* **2002**, *277*, 17962.
10. Duysen, E. G.; Li, B.; Xie, W.; Schopfer, L. M.; Anderson, R. S.; Broomfield, C. A.; Lockridge, O. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 528.
11. Chen, Y. S.; Chien, C. H.; Goparaju, C. M.; Hsu, J. T.; Liang, P. H.; Chen, X. *Protein Expr. Purif.* **2004**, *35*, 142.
12. Olsen, C.; Wagtmann, N. *Gene* **2002**, *299*, 185.
13. Chiravuri, M.; Agarraberes, F.; Mathieu, S. L.; Lee, H.; Huber, B. T. *J. Immunol.* **2000**, *165*, 5695.
14. Chiravuri, M.; Lee, H.; Mathieu, S. L.; Huber, B. T. *J. Biol. Chem.* **2000**, *275*, 26994.
15. Chiravuri, M.; Schmitz, T.; Yardley, K.; Underwood, R.; Dayal, Y.; Huber, B. T. *J. Immunol.* **1999**, *163*, 3092.
16. Danilov, A. V.; Klein, A. K.; Lee, H. J.; Baez, D. V.; Huber, B. T. *Br. J. Haematol.* **2005**, *128*, 472.
17. Shreder, K. R.; Wong, M. S.; Corral, S.; Yu, Z.; Winn, D. T.; Wu, M.; Hu, Y.; Nomanbhoy, T.; Alemayehu, S.; Fuller, S. R.; Rosenblum, J. S.; Kozarich, J. W. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4256.
18. Senten, K.; Van der Veken, P.; De Meester, I.; Lambeir, A. M.; Scharpe, S.; Haemers, A.; Augustyns, K. *J. Med. Chem.* **2003**, *46*, 5005.
19. Senten, K.; Van Der Veken, P.; De Meester, I.; Lambeir, A. M.; Scharpe, S.; Haemers, A.; Augustyns, K. *J. Med. Chem.* **2004**, *47*, 2906.
20. Ennis, M.; Hoffman, R.; Ghazal, N.; Old, D.; Mooney, P. *J. Org. Chem.* **1996**, *61*, 5813.
21. Hu, Y.; Ma, L.; Wu, M.; Wong, M. S.; Li, B.; Corral, S.; Yu, Z.; Nomanbhoy, T.; Alemayehu, S.; Fuller, S. R.; Rosenblum, J. S.; Rozenkrants, N.; Minimo, L. C.; Ripka, W. C.; Szardenings, A. K.; Kozarich, J. W.; Shreder, K. R. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4239.
22. Adherent (293T HEK) cells (0.58×10^6) were plated on a 6-well plate the day before the experiment. The next day media were changed and compounds or DMSO vehicle were added. After 3 h, cells were washed twice with PBS and harvested. 1×10^6 suspension cells (Jurkat) were seeded in a 24-well plate and incubated with inhibitors, after 3 h cells were washed twice with PBS. Cells were lysed in lysis buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 2 mM EDTA, 10 mM KCl, 0.1% Nonidet P-40, 5 μg/ml antipain, and 5 μg/ml leupeptin) for 30 min at 4 °C. DPP activity was measured using the fluorogenic substrate AP-AFC (2 mM in 50 mM Hepes buffer, pH 7.5) on an Fmax fluorescence plate reader (Molecular Devices, Menlo Park, CA) excitation, 410 nm; emission, 510 nm. The specificity of compounds was investigated by treating untransfected HEK cells with the same concentration of compound that inhibited approximately half of the overexpressed DPP II activity (293T HEK column).
23. 1×10^6 PBMCs in 0.5 ml of culturing media were plated in 24-well flat-bottomed plates in triplicate and treated with VbP at final concentration 10 μM DMSO (vehicle control), and AX8819 at 4.4, 8.8, 22, and 44 μM final concentrations. Cells were washed twice with PBS and were stained with 2 μl AnV—Allophycocyanin (APC) in 50 μl of Annexin V binding buffer for 15 min on ice, after that 350 μl FACS buffer (phosphate-buffered saline (PBS) supplemented with 1% FBS and 0.01% sodium azide) and PI (propidium iodide 10 μg/ml) were added. Experiments were repeated at least twice. PBMCs from different individuals were used to confirm the reproducibility of results.
24. Kuhn, H.; Weser, L.; Gessner, C.; Hammerschmidt, S.; Wirtz, H. *Oncol. Rep.* **2005**, *14*, 759.
25. Micheau, O.; Lens, S.; Gaide, O.; Alevizopoulos, K.; Tschopp, J. *Mol. Cell. Biol.* **2001**, *21*, 5299.
26. Maes, M. B.; Martinet, W.; Schrijvers, D. M.; Van der Veken, P.; De Meyer, G. R.; Augustyns, K.; Lambeir, A. M.; Scharpe, S.; De Meester, I. *Biochem. Pharmacol.* **2006**, *72*, 70.
27. Robertson, J. D.; Gogvadze, V.; Zhivotovsky, B.; Orrenius, S. *J. Biol. Chem.* **2000**, *275*, 32438.