Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 613–618

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

AN ENDOGENOUS SLEEP-INDUCING COMPOUND IS A NOVEL COMPETITIVE INHIBITOR OF FATTY ACID AMIDE HYDROLASE

Matthew P. Patricelli, Jean E. Patterson, Dale L. Boger,[a] and Benjamin F. Cravatt[b]*

Skaggs Institute for Chemical Biology and the Departments of [a]Chemistry, and [b]Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California, 92037, U.S.A.

Received 23 January 1998; accepted 2 February 1998

Abstract: 2-Octyl γ-bromoacetoacetate (OγBr), an endogenous compound originally isolated from human cerebrospinal fluid (CSF), has previously been demonstrated to increase REM sleep duration in cats. Based on the chemical structure of OγBr and its reported sleep-inducing effects, we synthesized OγBr along with chemically related analogs and tested these compounds as inhibitors of fatty acid amide hydrolase (FAAH), a brain enzyme that degrades neuromodulatory fatty acid amides. OγBr was found to competitively inhibit FAAH activity with IC_{50} and K_i values of 2.6 μM and 0.8 μM, respectively [for the (*R*)-enantiomer of OγBr (**1**)]. A set of synthetic analogs of OγBr was examined to define the structural features required for FAAH inhibition and inhibitor potencies were assessed at pH 9.0 (near the pH optimum of FAAH) and pH 7.0. Interestingly, at pH 7.0 the γ-halo β-keto ester inhibitors proved to be significantly more potent than the trifluoromethyl ketone of oleic acid, one of the most potent FAAH inhibitors described to date. This study supports the possibility that OγBr may be a physiological regulator of FAAH activity and fatty acid amide levels in vivo. Additionally, the characterization of γ-halo β-keto esters as powerful FAAH inhibitors near physiological pH may aid in future studies of the enzymology and biological properties of FAAH.

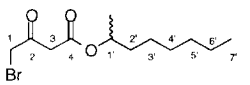
Fatty acid amides represent a growing family of biologically active lipids implicated in a diverse range of cellular and physiological processes.[1-3] Members of the fatty acid amide family include anandamide (arachidonoyl ethanolamide), an endogenous ligand for the CB1 cannabinoid receptor[2] and oleamide [9(*Z*)octadecenamide], a sleep-inducing compound isolated from the cerebrospinal fluid of sleep-deprived cats.[1,4] Consistent with its proposed role as an endogenous cannabinoid, anandamide has been shown to elicit several physiological effects common to cannabinoid agonists.[5] Oleamide has been shown to act in a structurally specific manner to induce physiological sleep in rats[1] and potentiate 5-HT receptor responses to serotonin.[6,7] Both anandamide[8] and oleamide[9] have been found to block gap junction communication in glial cells.

Anandamide and oleamide are rapidly degraded to their corresponding inactive acids by a membrane-bound enzyme activity, fatty acid amide hydrolase (FAAH).[10] Recent studies have suggested that manipulation of FAAH activity in vivo can affect both the level and duration of the responses elicited by its fatty acid amide substrates. Addition of FAAH inhibitors to neuroblastoma cell cultures increased the amounts of anandamide released,[11] and FAAH-resistant analogs of anandamide have been shown to induce prolonged inhibition of motor activity.[12] We identified 2-octyl γ-bromoacetoacetate (OγBr, **1**), an endogenous compound originally isolated from human cerebrospinal fluid,[13] as a possible FAAH regulator based on its physiological and chemical properties. OγBr has been found in rat cortex, pituitary, and retinal tissues,[14] and was shown to induce REM

*Corresponding Author
email: cravatt@scripps.edu

0960-894X/98/$19.00
PII: S0960-894X(98)00073-0

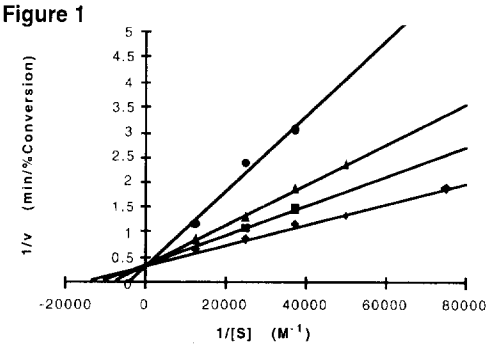
sleep in cats.[15] The chemical features of OγBr, including an extended hydrophobic alkyl chain and a γ-bromo β-keto ester substituent capable of potential active site alkylation, suggested that this compound may act as a FAAH inhibitor. Therefore, we synthesized OγBr as well as several related analogues and tested these agents as potential FAAH inhibitors.



2-Octyl γ-bromoacetoacetate (OγBr)

Compounds **1**-**5** and **7**[16] were anticipated to be irreversible inactivators of FAAH based on numerous previous studies documenting the active site alkylation of proteases by α-halo ketones.[17] However, the kinetics of inhibition observed for these compounds over the reaction time courses monitored (30 s to 15 min at pH 9.0 and 10 min to 1 h at pH 7.0) appeared linear. Additionally, Lineweaver-Burk plot analysis of FAAH inhibition by **1** suggested competitive inhibition (Figure 1). To determine the extent of reversibility of these agents under the experimental conditions, the inhibitors were pre-incubated with the FAAH preparation at pH 9.0 for various times before passing the mixture over a DEAE sepharose anion exchange column to remove reversibly bound inhibitor, and eluting FAAH activity with high salt (500 mM NaCl). A slight degree of irreversible inhibition (20-30% enzyme inactivated at 5 μM inhibitor concentration) was detectable after 15 min (the longest time point measured at pH 9.0) in the case of agents **1** and **3**, however this level of enzyme inactivation did not appear to significantly affect the reaction progress curves or the measured K_i values for these two compounds. After pre-incubations greater than 2 h, inhibitors **1**-**5** and **7** all exhibited significant (between 20-30%) irreversible inhibition of FAAH activity.

Figure 1

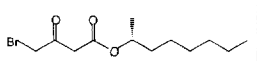
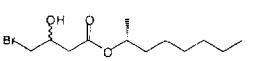
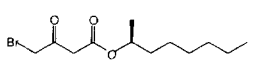
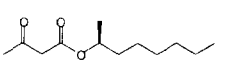
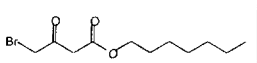
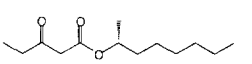
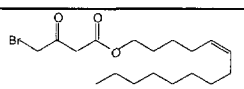
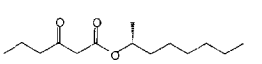
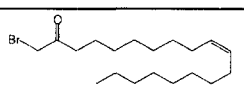
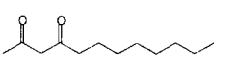
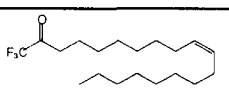
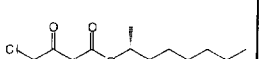


Lineweaver-Burk plot of competitive FAAH inhibition by **1**. FAAH activity was monitored in the presence of 0 μM (◆), 0.5 μM(■), 1 μM (▲), and 2.5 μM (●) of **1** with oleamide concentrations from 13-80 μM.

The K_i value of **1** determined by Lineweaver-Burk and Dixon plot analysis was found to be 0.82 μM.[18] As detailed in Table 1, the K_i values for **1**-**7** (determined by Dixon plot analysis) varied less than one order of magnitude. Given that **6** was the most potent reversible FAAH inhibitor reported to date,[19] all of these compounds

represent effective FAAH inhibitors. Inversion of the C-1′ methyl substituent of **1** did not significantly affect inhibitory potency (**2**, K_i = 1.1 μM) while removal of this methyl group slightly increased potency (**3**, K_i = 0.55 μM). Replacement of the C-1 bromine moiety with chlorine did not greatly impact the observed K_i value (**7**, K_i = 1.1 μM). A significantly reduced K_i was found for **4** (K_i = 0.19 μM), a γ-bromo β-keto ester designed to mimic the structure of oleamide. Inhibitor **4** proved similar in potency to the trifluoromethyl ketone of oleic acid (**6**, K_i = 0.25 μM) and slightly more potent than the α-bromo ketone of oleic acid (**5**, K_i = 0.39 μM). Removal of the C-1 bromine substituent of **1** (**9**), replacement of this halogen with methyl (**10**) or ethyl (**11**) groups, or reduction of the C-2 carbonyl (**8**) all produced inactive compounds showing no detectable inhibition at concentrations as high as 100 μM. Agent **12** which possesses similar enolization properties to those of the γ bromo β-keto esters[20] was also void of inhibitory activity. These results strongly suggest that an electron-deficient carbonyl at the C-2 position is required for the inhibitory potency of the γ-bromo β-keto ester inhibitors.

Table 1. Potency of Inhibitors at pH 9.0[a]

	Agent	IC_{50} (μM) at pH 9.0	K_i (μM) at pH 9.0		Agent	IC_{50} (μM) at pH 9.0	K_i (μM) at pH 9.0
1		2.6 ± 0.5	0.8 ± 0.1	**8**		>100	ND
2		4.0 ± 0.6	1.1 ± 0.3	**9**		>100	ND
3		1.5 ± 0.1	0.55 ± 0.04	**10**		>100	ND
4		0.6 ± 0.1	0.19 ± 0.03	**11**		>100	ND
5		1.00 ± 0.05	0.39 ±0.02	**12**		>100	ND
6		0.6 ± 0.1[b]	0.25 ± 0.02				
7		2.9 ± 0.2	1.10 ± 0.08				

[a]All enzyme assays (except where noted) were performed between 20–22 °C using a solubilized liver plasma membrane extract in a reaction buffer of 125 mM Tris, 1 mM EDTA, 0.4.% glycerol, 0.04% Triton X-100, pH 9.0 buffer. Activity was monitored by following the breakdown of ^{14}C-oleamide to oleic acid after extraction and separation by thin-layer chromatography. Radioactivity was quantitated by scintillation counting.
[b]Agent **6** exhibited slow binding kinetics and the K_i value reported here reflects the inhibition observed at equilibrium.

Table 2. Potency of Inhibitors at pH 7.0

Inhibitor	IC_{50} (μM) at pH 7.0
1	3.1 ± 0.2
3	2.1 ± 0.6
4	2.5 ± 0.4
5	5.3 ± 0.4
6	9.9 ± 0.7
7	0.7 ± 0.1

To further test the possibility that **1** could act as a physiological inhibitor of FAAH we determined the relative potencies of several FAAH inhibitors at pH 7.0 (Table 2).[21] The most drastic change in inhibitor potency was observed for the trifluoromethyl ketone inhibitor **6**, which was one of the most potent inhibitors at pH 9.0. Agent **6** suffered a 17-fold increase in IC_{50} from pH 9.0 to pH 7.0 becoming the least active inhibitor at pH 7.0. In direct contrast to **6**, inhibitor **7** exhibited a significant increase in potency from pH 9.0 to pH 7.0 making it the most potent inhibitor at pH 7.0. The potencies of **4** and **5** decreased approximately five–fold from pH 9.0 to pH 7.0, while **1** and **3** exhibited relatively minor decreases in potency over this pH range. Overall, the pH-dependency of the FAAH inhibitors **1**–**7** underscores an intriguing observation that as physiological pH is approached, the natural compound **1** and closely related analogs **3**, **4**, and **7** become the most potent FAAH inhibitors tested, surpassing the trifluoromethyl ketone **6** by as much as 14-fold in potency. These results support the possibility that **1** could serve as an endogenous regulator of FAAH activity.

In this study several analogues of the natural human brain constituent, 2-octyl γ-bromoacetoacetate (OγBr), employing a γ-halo β-keto ester moiety were found to be potent inhibitors of FAAH. At pH 9.0, agent **4** proved as potent as the the most potent reversible FAAH inhibitor described to date (**6**), while at pH 7.0, all of the γ-halo β-keto ester agents surpassed the potency of agent **6**. The relatively high potency of OγBr at physiological pH, as well as its tissue distribution and sleep-inducing properties support the possibility that OγBr may act as an endogenous FAAH regulator. In this regard, OγBr, like the fatty acid amides, oleamide[22] and anandamide,[5] has recently been found to induce hypothermia in rats.[22] The more potent analogues of OγBr presented here may prove useful as research tools or therapeutic agents active at physiological pH.

Acknowledgments. We gratefully acknowledge the financial support of the National Institutes of Health (MH58542, BFC; CA42056, DLB), the Skaggs Institute for Chemical Biology (BFC, DLB) and the National Science Foundation (predoctoral fellowship, MPP). We would also like to thank Edouard Lerner for providing compound **6**, Ian Ollman and James LaClair for suggestions in preparing the manuscript, and Dan Giang for technical assistance.

References and Notes

1. Cravatt, B. F.; Prospero-Garcia, O.; Siuzdak, G.; Gilula, N. B.; Henriksen, S. J.; Boger, D. L.; Lerner, R. A. *Science* **1995**, *268*, 1506.

2. Devane, W. A.; Hanus, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. *Science* **1992**, *258*, 1946.

3. (a) Facci, L.; Dal Tosso, R.; Romanello, S.; Buriani, A.; Skaper, S. D.; Lean, A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3376. (b) Wakamatsu, K.; Masaki, T.; Itoh, F.; Koichi, K.; Sudo, K. *Biochem. Biophys. Res. Commun.* **1990**, 423. (c) Ratnayake, S.; Fang, X. P.; Anderson, J. E.; McLaughlin, J. L.; Evert, D. R. *J. Nat. Prod.* **1992**, *55*, 1462.

4. Lerner, R. A.; Siuzdak, G.; Prospero-Garcia, O.; Henriksen, S. J.; Boger, D. L.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9505.

5. Smith, P. B.; Compton, D. R.; Welch, S. P.; Razdan, R. K.; Mechoulam, R.; Martin, B. R. *J. Pharmacol. Exp. Ther.* **1994**, *270*, 219.

6. Huidobro-Toro, J.; Harris, R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8078

7. Thomas, E. A.; Carson, M. J.; Neal, M. J.; Sutcliffe, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14115.

8. Venance, L.; Piomelli, D.; Glowinski, J.; Giaume, C. *Nature* **1995**, *376*, 590.

9. Guan, X.; Cravatt, B. F.; Ehring, G. R.; Hall, J. E.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *J. Cell Biol.* **1997**, *139*, 1785.

10. (a) Cravatt, B. F.; Giang, D. K.; Mayfield, S. P.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *Nature* **1996**, *384*, 83. (b) Chin, S.; Deutsch, D. G. *Biochem. Pharm.* **1993**, *46*, 791. (c) Giang, D. K.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2238. (d) Maurelli, S.; Bisogno, T.; De Petrocellis, L.; Di Luccia, A.; Marino, G.; Di Marzo, V. *FEBS Lett.* **1995**, *377*, 82.

11. Koutek, B.; Prestwich, G. D.; Howlett, A. C.; Chin, S. A.; Salehani, D.; Akhavan, N.; Deutsch, D. J. *J. Biol. Chem.* **1994**, *269*, 22937.

12. Romero, J.; Garcia-Palmero, E.; Lin, S. Y.; Ramos, J. A.; Makriyannis, A.; Fernandez-Ruiz, J. J. *Life Sci.* **1996**, *58*, 1249.

13. Yanagisawa, I.; Yoshikawa, H. *Biochim. Biophys. Acta* **1973**, *329*, 283.

14. Yamane, M.; Maeda, K.; Yanagisawa, I. *Sleep Res.* **1980**, *9*, 60.

15. Torii, S.; Mitsumori, K.; Inubushi, S.; Yanagisawa, I. *Psychopharmacologia* **1973**, *29*, 65.

16. Compounds **1**–**4**, **7**, and **9** were synthesized as described by Yanigisawa and Yoshikawa[13] with the following modifications. 5(*Z*)-Tetradecenol[23] was used in the synthesis of agent **4**. Agent **7** was prepared using Cl_2 in place of Br_2 at 25 °C instead of 0 °C. Compounds **5** and **6** were synthesized as described[18] except that in the case of **5**, Br_2 was substituted for Cl_2. Compound **8** was prepared from **1** by reduction with $NaBH_4$ in EtOH as described.[24] Agents **10** and **11** were synthesized using the methodology of Streiber and Zibuck.[25] Agent **12** was prepared according to the method described by Swamer and Hauser.[26]

17. (a) Bender, M. L.; Brubacher, L. J. *J. Am. Chem. Soc.* **1966**, *88*, 5880. (b) Daniels, S.; Katzenellenbogen, J. *Biochemistry* **1986**, *25*, 1436. (c) Silverman, R. B. *Mechanism Based Enzyme*

Inactivation; CRC Press, Boca Raton, 1988; Vol. 1. (d) Page, M. I. In *Comprehensive Medicinal Chemistry*; Sammes P. G., Ed.; Pergamon, Oxford; 1990; Vol. 2.

18. Results from Lineweaver–Burk plot analysis of FAAH in the presence of varying oleamide concentrations indicated a K_m value for oleamide of 31 ± 3 μM. This K_m value was somewhat higher than the K_m values of 5,[19] 9,[10d] and 14 μM,[10d] obtained in previous studies of FAAH. While these investigations used similar membrane preparations, none of these efforts studied detergent-solubilized FAAH activities. One previous analysis of FAAH activity in a solubilized system also obtained a significantly higher K_m value for anandamide (60 μM)[27a] than values reported elsewhere (3.4[27b] and 12.7 μM[27c]).

19. Patterson, J. E.; Ollmann, I. R.; Cravatt, B. F.; Boger, D. L.; Wong, C.-H.; Lerner, R. A. *J. Am. Chem. Soc.* **1996**, *118*, 5938.

20. **1**-**4** were observed to exist to a significant extent as enols (36% in $CDCl_3$) by ^1H NMR and their pK_a values were close to the pH at which they were tested (pK_a of **1** = 8.5). In contrast, agents lacking the C-1 bromine substituent were found to have much higher pK_a values (pK_a of **9** = 10.5). The β-diketone **12** was found to have a pK_a value of 9.0, and existed almost exclusively as an enol (in $CDCl_3$) by ^1H NMR.

21. At pH 7.0, longer reaction time courses (up to 1 h) were needed as FAAH activity was significantly (approximately six–fold) slower at this pH. Although the reaction progress curves for all compounds tested at pH 7.0 appeared linear, significant amounts of alkylation were detected by the above described method after 1 h for agents **1** and **3** (up to 60% inactivation by agents **1** and **3** at 5 μM). It is at present not clear why the inactivation exhibited by these compounds is not reflected in the reaction progress curves, and therefore based on the apparent complexity underlying the inhibition kinetics at longer reaction times, we are only reporting IC_{50} values with this data.

22. Henriksen, S. J.; Horn, T. Unpublished observations.

23. Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580.

24. Kato, T.; Kimura, H. *Chem. Pharm. Bull.* **1977**, *25*, 2692.

25. Zibuck, R.; Streiber, J. *Org. Syn.* **1993**, *71*, 236.

26. Swamer, F. W.; Hauser, C. R. *J. Am. Chem. Soc.* **1950**, *72*, 1352.

27. (a) Ueda, N.; Kurahashi, Y.; Yamamoto, S.; Tokunaga, T. *J. Biol. Chem.* **1995**, *270*, 23823. (b) Hillard, C. J.; Wilkinson, D. M.; Edgemond, W. S.; Campbell, W. B. *Biochim. Biophys. Acta.* **1995**, *1257*, 249. (c) Desarnaud, F.; Cadas, H.; Piomelli, D. *J. Biol. Chem.* **1995**, *270*, 6030.