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Correlation of inhibitor effects on enzyme activity and thermal stability for the integral membrane protein fatty acid amide hydrolase

Ian M. Slaymaker [a], Michael Bracey [b], Mauro Mileni [a], Joie Garfunkle [c], Benjamin F. Cravatt [b,d], Dale L. Boger [c,d], Raymond C. Stevens [a,*]

[a] Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[b] Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[c] Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[d] Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

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ABSTRACT

The melting curves of fatty acid amide hydrolase (FAAH) in the presence of 29 reversible inhibitors were measured using a thiol-reactive fluorophore. The thermal stability (T_m) of the FAAH/inhibitor complex varied significantly depending on the chemical characteristics of the inhibitors, notably variations in the head group. Two separate distributions were observed when T_m was plotted against K_i. The majority of the inhibitors showed a positive correlation between binding affinity and T_m, however inhibitors with a pyridine carboxylic acid moiety in the head group fell in a distinct and uncorrelated distribution when tail groups were varied.

It has been shown that the effects of ligands on an enzyme may be observed by measuring shifts in the enzyme's thermal stability, which may in turn have implications on inhibitor potency. Indeed, a potential correlation between thermal stability and ligand affinity has been observed.[1] An additional motivation for studying the effect of ligand binding on stability is its implications toward crystallization of protein–ligand complexes since thermal stability has been linked to an increased likelihood of improving crystal diffraction.[2]

Fatty acid amide hydrolase (FAAH) is an integral membrane protein and the primary enzyme responsible for the catabolism of fatty acid amides in vivo including anandamide, an endogenous cannabinoid, and oleamide, a sleep-inducing signaling molecule.[3] FAAH inactivation has been shown to reduce pain and inflammation,[4,5] which makes the enzyme a potential new drug target for possible analgesics. Consistent with this premise, the FAAH inhibitors URB597 and OL-135 (compound **16** in the present study) have shown in vivo efficacy in mouse pain models.[6,7] The structure of rat FAAH was solved at 2.8 Å in complex with the irreversible covalent inhibitor methoxy arachidonyl fluorophosphonate (MAFP),[8] but additional structures with pharmacologically relevant inhibitors remain to be elucidated. The capacity to rank new compounds by their ability to impart structural stability may prove to be a key in obtaining new FAAH–inhibitor structures, thereby furthering our understanding of the enzyme's mechanism and facilitating drug design.

Mei et al.[9] studied the stability of FAAH as a function of guanidinium hydrochloride concentration and hydrostatic pressure and concluded that conformational changes mediated by inhibitor binding to the active site lead to tighter interaction between monomers and an increase in enzyme stability. This also resulted in a reduced ability of the protein to bind to membranes. In a more high throughput manner and with an increased array of inhibitors, we report a survey of a representative library of reversible inhibitors that has been created based on the enzyme's natural substrates.[10] To date, these compounds have been ranked as inhibitors based primarily on K_i.[11–15] We explore the relationship between K_i and stability, and the potential use of protein melting point as a complementary metric for assessing ligand efficacy for crystallization and drug candidate screening. Using a thiol-reactive fluorophore, we determined melting curves for FAAH either alone or in the presence of a variety of inhibitors. Relationships between T_m and K_i are discussed.

The identification of suitable enzyme inhibitors is a focus of many areas of protein biochemistry including structural studies and drug design. A leading parameter often cited for ranking small molecules is their inhibitory potency as measured enzymologically by IC_{50} or K_i. However, as these approaches are valuable, they may prove to be insufficient for gaining a comprehensive understanding of potential inhibitor–enzyme complexes. For example, the measurement of the on–off-rates or the stabilizing potential of lead

* Corresponding author. Tel.: +1 858 784 9416; fax: +1 858 784 9483.
E-mail address: stevens@scripps.edu (R.C. Stevens).

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compounds may provide complementary yet independent insight into molecular interactions. We have taken the latter approach to characterize 29 compounds (in addition to the co-crystallized irreversible inhibitor MAFP) against the integral membrane enzyme FAAH by measuring the protein's melting point (T_m) once complexed with these inhibitors using CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin) as an indicator (Supplementary Material).[16] As temperature increases, domains of the protein unfold to expose internal cysteines to the CPM in the solvent. Fluorescence increases as CPM in the solvent binds to the newly exposed cysteines, indicating that the protein is unfolding, and allowing T_m to be measured. In comparing these results with predetermined K_i values, we find that correlations between the two approaches differ depending on the structural characteristics of the inhibitors themselves.

The inhibitors tested in the present study can be described as bivalent with a hydrophilic 'head group' that likely binds to the enzyme's catalytic triad and cytosolic port and a hydrophobic 'tail group' that packs in the enzyme's acyl chain binding pocket.[8] In characterizing the effects of these compounds, two distinct distributions become apparent when stability (T_m) is plotted against inhibition (K_i) (Fig. 1). One distribution demonstrates a strong linear correlation between stability and binding affinity ($R^2 = 0.81$) and comprises a varied collection of head and tail group structures (Supplemental Fig. 1). In contrast, a second distribution (circled) is distinguished by a constant head group (2-pyridine-6-carboxylic acid) with variable hydrophobic tails (Fig. 1). This group exhibits a less clear correlation between stability and inhibition. In this study, **1** (Fig. 2) was identified as the most stabilizing inhibitor for FAAH despite having an intermediate K_i.

The pyridine nitrogen in the correlated and uncorrelated classes of inhibitors has been shown to be an important factor for inhibitor affinity and has been attributed to hydrogenbond formation in the active site and cytosolic port of FAAH.[11] The addition of a carboxylic acid to this head group results in a significant increase in stability compared to the addition of alternative functionalities (Fig. 3). An increase in stability is observed for all inhibitors surveyed that contain the 2-pyr-6-CO_2H portion, validating the observation that this group is an important stabilizing factor (Fig. 4).

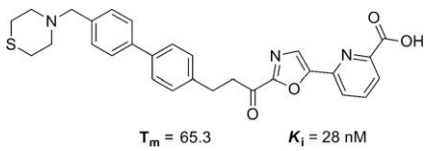

Figure 2. Most thermally stabilizing inhibitor: compound **1**. By comparison, the T_m for MAFP treated protein, which yielded the only available crystal structure, is 59.3.

However, it was also observed that this modification led to a decrease in inhibitor potency. One possible explanation for this counterintuitive observation is that the on-rate may be reduced for carboxylic acid-bearing inhibitors, and they interact more tightly once bound. Attempts to determine the on- and off-rates of any FAAH compound by surface plasmon resonance have not been successful due to the incompatible nature of this particular system (the presence of detergent, extreme hydrophobicity and low molecular weight of the ligands, etc.). Alternatively, since the enzymatic assay is conducted at pH 9, where the carboxylic acids are most likely deprotonated, while the thermal denaturations are conducted at pH 7.5, it is possible that the enzymatic assays underestimate the inhibitory potency and binding affinity at physiological pH. Indeed, in the case of **2**, the K_i as measured at pH 7.5 is 10.4 nM as opposed to 20 nM at pH 9.[14]

To probe if the importance of the carboxylic acid head group lies in its interaction with the catalytic base Lys142, mutant rat FAAH K142A was tested against **16** and its carboxylic acid-bearing analog **2** (Fig. 4). Though these compounds yield different K_i values toward wild-type enzyme, they each provided indistinguishable stabilizing potential toward both the wild-type and mutant proteins (Fig. 5). Apo T_m in this study varies slightly from that indicated in Figure 1 due to the K142A mutant only being available in a slightly different construct and purification method. This indicates that the interaction that imparts increased stabilizing potential to the carboxylic acid-bearing compounds must lie with some other amino acid in the protein. Monte Carlo simulations suggest that there are other candidates available for such hydrogen-bond formation.[17]

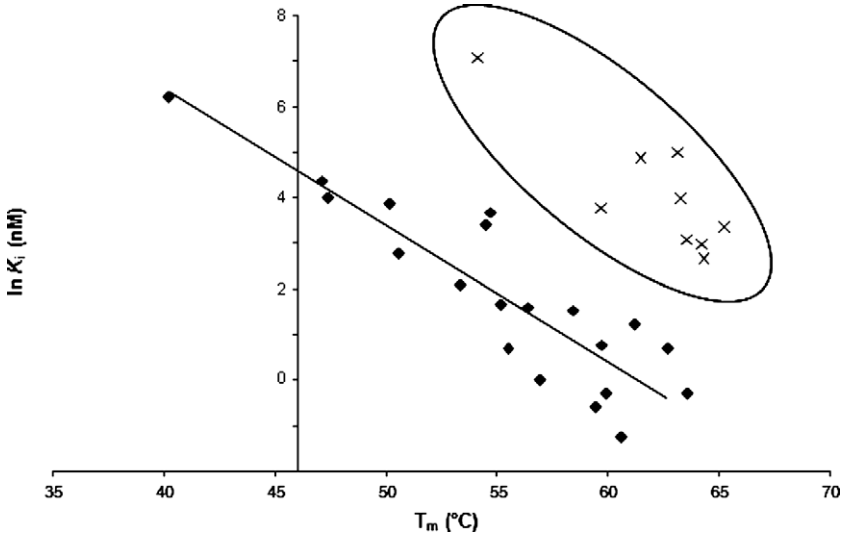

Figure 1. T_m versus ln(K_i) showing two distinct distributions of inhibitors with (×) or without (♦) a 2-pyridine-6-carboxylic acid head groups. The former inhibitors are distributed with no apparent correlation between stability and inhibition (oval). Remaining inhibitors show a strong correlation between T_m and K_i ($R^2 = 0.81$). The *Y*-axis intersects the *X*-axis at the T_m for the apo-enzyme. No K_i value was attainable for the 2-pyr-6-CO_2H compound **9**, although thermal stability was substantially increased (T_m = 61.4). MAFP is not shown on the graph due to its irreversible binding, thus a lack of K_i (T_m = 59.3). Efforts to fit a regression line for those inhibitors bearing pyridine-6-carboxylic acid head groups (×) yielded poor fit ($R^2 = 0.72$) strongly dominated by outlier compound **23**. Omitting this latter compound from the regression yields no fit ($R^2 = 0.24$).

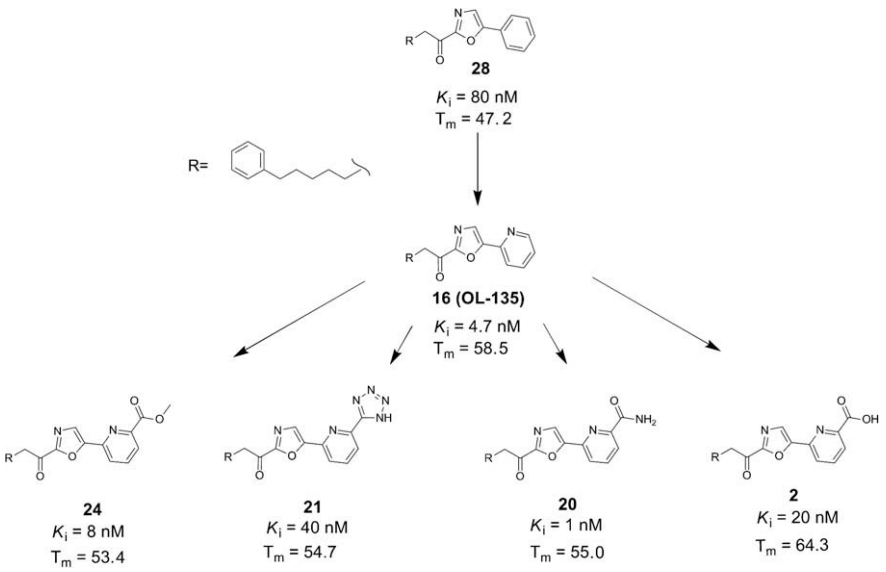

Figure 3. Resulting changes to K_i and T_m by the addition of a nitrogen to the aryl head group and subsequent extensions of the pyridine.

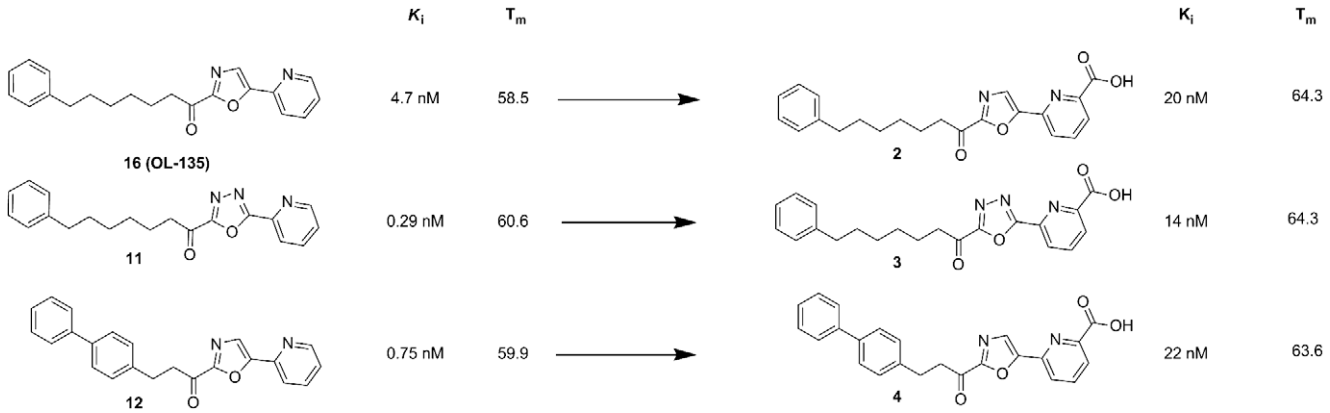

Figure 4. The addition of a carboxylic acid significantly increases stability (T_m) but lowers inhibitory potency (K_i).

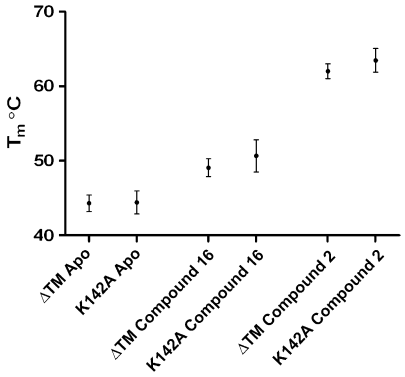

Figure 5. K142A rat FAAH showed no difference in stability when bound to compound **16** or its carboxylic acid-bearing analog **2** compared to wild-type ΔTM rat FAAH expressed from the pTrcHis vector[18] (see Supplemental Material).

In conclusion, this study shows a correlation between inhibitory potency and stabilizing potential of a class of FAAH inhibitors that bear a variety of hydrophilic head groups that are poised to interact with the catalytic triad and cytosolic port of the enzyme. In contrast, when the head group is held constant and a variety of hydrophobic tail groups are examined there is no clear correlation. These results may arise from the differences in the energetics of binding attributed to the hydrophobic interactions in the acyl binding pocket versus the more polar and complementary ones in the cytosolic port of the enzyme. Additionally, the introduction of a carboxylic acid to the aryl head group of an inhibitor is not only more stabilizing, but also creates an independent relationship to inhibitory potency when compared to other inhibitors. The question of how the carboxylic acid is behaving in the binding pocket of FAAH, whether the inhibitors are binding in a different mode (protonated vs deprotonated), and what specific interaction may be responsible remains to be established. These observations made using the thermal denaturation assay provide a complementary approach to evaluate candidate ligands providing insights not observed using more classical methods.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.086.

References and notes

1. Matulis, D.; Kranz, J. K.; Salemme, F. R.; Todd, M. J. *Biochemistry* **2005**, *44*, 5258.
2. Ericsson, U. B.; Hallberg, B. M.; Detitta, G. T.; Dekker, N.; Nordlund, P. *Anal. Biochem.* **2006**, *357*, 289.
3. McKinney, M. K.; Cravatt, B. F. *Annu. Rev. Biochem.* **2005**, *74*, 411.
4. Lichtman, A. H.; Shelton, C. C.; Advani, T.; Cravatt, B. F. *Pain* **2004**, *109*, 319.
5. Cravatt, B. F.; Saghatelian, A.; Hawkins, E. G.; Clement, A. B.; Bracey, M. H.; Lichtman, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10821.
6. Lichtman, A. H.; Leung, D.; Shelton, C.; Saghatelian, A.; Hardouin, C.; Boger, D.; Cravatt, B. F. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 441.
7. Russo, R.; Loverme, J.; La Rana, G.; Compton, T. R.; Parrott, J.; Duranti, A.; Tontini, A.; Mor, M.; Tarzia, G.; Calignano, A.; Piomelli, D. *J. Pharmacol. Exp. Ther.* **2007**, *322*, 236.
8. Bracey, M. H.; Hanson, M. A.; Masuda, K. R.; Stevens, R. C.; Cravatt, B. F. *Science* **2002**, *298*, 1793. PDB Accession No. 1MT5.
9. Mei, G.; Di Venere, A.; Gasperi, V.; Nicolai, E.; Masuda, K. R.; Finazzi-Agro, A.; Cravatt, B. F.; Maccarrone, M. *J. Biol. Chem.* **2007**, *282*, 3829.
10. Boger, D. L.; Sato, H.; Lerner, A. E.; Hedrick, M. P.; Fecik, R. A.; Miyauchi, H.; Wilkie, G. D.; Austin, B. J.; Patricelli, M. P.; Cravatt, B. F. *Proc Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5044.
11. Romero, F. A.; Du, W.; Hwang, I.; Rayl, T. J.; Kimball, F. S.; Leung, D.; Hoover, H. S.; Apodaca, R. L.; Breitenbucher, J. G.; Cravatt, B. F.; Boger, D. L. *J. Med. Chem.* **2007**, *50*, 1058.
12. Hardouin, C.; Kelso, M. J.; Romero, F. A.; Rayl, T. J.; Leung, D.; Hwang, I.; Cravatt, B. F.; Boger, D. L. *J. Med. Chem.* **2007**, *50*, 3359.
13. Kimball, F. S.; Romero, F. A.; Ezzili, C.; Garfunkle, J.; Rayl, T. J.; Hochstatter, D. G.; Hwang, I.; Boger, D. L. *J. Med. Chem.* **2008**, *51*, 937.
14. Garfunkle, J.; Ezzili, C; Rayl, T. J.; Hochstatter, D. G.; Hwang, I.; Boger, D. L. *J. Med. Chem.* in press.
15. Boger, D. L.; Miyauchi, H.; Du, W.; Hardouin, C.; Fecik, R. A.; Chang, H.; Hwang, I.; Hedrick, M. P.; Leung, D.; Acevedo, O.; Guimaraes, C. R. W.; Jorgensen, W. L.; Cravatt, B. F. *J. Med. Chem.* **2005**, *48*, 1849.
16. Alexandrov, A. I.; Mileni, M.; Chien, E. Y. T.; Hanson, M. A.; Stevens, R. C. *Structure* **2008**, *16*, 351.
17. Guimaráes, C. R. W.; Boger, D. L.; Jorgensen, W. L. *J. Am. Chem. Soc.* **2005**, *127*, 17377.
18. Patricelli, M. P.; Lashuel, H. A.; Giang, D. K.; Kelly, J. W.; Cravatt, B. F. *Biochemistry* **1998**, *37*, 15177.