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Multiplexed Gel-based ABPP Strategy for Target-agnostic Serine Hydrolase Inhibitor Screening

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Abstract: Serine hydrolases (SHs) represent a functionally defined enzyme class with significant roles in human physiology, yet many of the approximately 240 human SH's lack selective chemical inhibitors required for functional validation. To address this, we developed a medium throughput screening platform based on multiplexed gel-based activity-based protein profiling (ABPP). Using mouse brain proteome as a source, we performed a target-agnostic screen of 1,664 covalent compounds, leading to the identification of three micromolar-potent fatty acid amide hydrolase (FAAH) inhibitors featuring urea warheads and one epoxide-based inhibitor targeting an unannotated protein. An α/β –hydrolase domain-containing protein 2 (ABHD2) interaction was identified *via* chemical proteomics, which was validated with overexpression studies in U2OS cells. While the current approach is biased toward metabolic SHs and constrained by gel resolution, it provides a scalable workflow to facilitate the discovery of selective hits and mechanism-of-action studies for underexplored proteins.

Keywords: ABHD2 · Activity-based protein profiling · FAAH; Hit-finding · Serine hydrolase

1. Introduction

Serine hydrolases (SHs) constitute an enzyme class that is defined by their catalytic function and play important roles in clinical applications. They exhibit substantial genetic and structural diversity. SHs lack a single conserved fold or common evolutionary origin, and instead share only a catalytic mechanism that relies on a nucleophilic serine residue to hydrolyse amide or (thio)ester bonds *via* a covalent acyl-enzyme intermediate.[1,2] Accordingly, their classification is based solely on function, and the approximately 240 human SHs are distributed across multiple superfamilies. They can be further distinguished by substrate preference: roughly 125 function as serine proteases (SPs), which cleave peptide backbones in proteins and predominantly adopt chymotrypsin- or subtilisin-like folds, whereas the remaining ~115 hydrolyse metabolites or reverse post-translational modifications.[3] This latter group is therefore commonly referred to collectively as the metabolic Serine Hydrolases (mSHs).

The involvement of mSHs in numerous physiological processes, most notably in digestion[4,5] as well as cholinergic[6] and endocannabinoid[7] signalling, makes them highly attractive targets for therapeutic intervention. Despite this clinical relevance, only a few market-approved drugs currently target mSHs.[1] Orlistat (tetrahydrolipstatin, THL) inhibits digestive lipases, such as gastric triacylglycerol lipase (LIPF) and pancreatic triacylglycerol lipase (PNLIP), and is used for the treatment of obesity.[8] The other two clinically validated mSH targets are acetylcholinesterase (ACHE) for the treatment of Alzheimer's disease,[9] and dipeptidyl peptidase 4 (DPP4), for the treatment of type 2 diabetes.[10,11] For many mSHs, however, no selective inhibitors are available to study their functions. There is therefore a strong need for such tool compounds to enable a better understanding of the roles that mSHs play in health and disease.

In earlier work in this field, Bachovchin *et al*. showed that a large majority (approximately 80%) of mSHs can be labelled by the archetypical fluorophosphonate (FP) activity-based probe.[12] Broad-spectrum chemical probes enable simultaneous readout of enzyme activity across large parts of the SH enzyme class, thereby allowing concurrent assessment of inhibitor potency and selectivity.[13] Typical SH inhibitor designs incorporate a mild electrophile into a non-covalent recognition motif, enabling covalent reaction with the nucleophilic serine residue. Electrophilic warheads commonly used against serine hydrolases include ureas,[14] cyano amides,[15] ketones,[16] and carbamates.[17,18] This covalent mode of action, together with the acyl-enzyme intermediate intrinsic to the SH catalytic mechanism, allows such inhibitors to be interrogated using activity-based protein profiling (ABPP).[19]

In this work, we expand the set of fluorophosphonate probes and establish a multiplexed gel-based ABPP assay to enable medium throughput screening of covalent fragments. Using this platform, we performed a target-agnostic screen of a serine hydrolase-focused compound library comprising more than 1600 members, which led to the identification of micromolar potent inhibitors of FAAH and ABHD2.

2. Results and Discussion

2.1 *Multiplexed Screening of a Serine Hydrolase-focused Covalent Compound Library*

To enable a multiplexed gel-based serine hydrolase screen, we required three different fluorescent probes (**1–3**). To this end, building on two previously reported fluorophosphonate probes (**1** and **2**) bearing BODIPY and TAMRA fluorophore, respectively,[20] we synthesised an additional Cy5-labelled variant (probe **3**) (Fig. 1A and Scheme 1 (SI)).

Mouse brain lysates were selected as the protein source, as this is a well-characterised system containing at least eight previously annotated mSH bands, including Pnpla6, Faah, Nceh1, Abhd12, Magl, Abhd6, and Lypla1 and Lypla2, as well as a variable number (>10) of additional, unannotated protein bands.[20] Lysates were first incubated separately with individual inhibitors from the

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library, followed by labelling with one of the three probes. After quenching, the samples were combined and analysed in a single sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) lane, enabling the simultaneous resolution of three different inhibitors per lane (Fig. 1B). Using standard 15-well gels, with one lane reserved for a protein marker and one for a positive control, this workflow enables the screening of 39 compounds on a single gel (n = 1).

To identify new serine hydrolase inhibitors, a serine hydrolase-focused compound library from Enamine was acquired. This library comprises 12,560 molecules bearing mildly electrophilic groups capable of covalently interacting with a nucleophilic serine residue. These electrophiles include ureas,[14] epoxides,[21] and ketones.[16] A subset of these compounds was selected based on the nature of the electrophilic warhead (Fig. 2A). To enrich the large class of urea-containing molecules, compounds were filtered for the presence of an aromatic heterocycle at the alpha-position of the urea group, a feature expected to increase the intrinsic reactivity to the urea moiety. This selection yielded 823 urea-containing compounds. In addition, all ketone-containing compounds lacking other electrophiles were included, yielding 441 compounds. Finally, all 400 epoxide-containing compounds were included, as epoxides are well known to be reactive towards cysteine residues,[21–24] and can also react with glutamate residues.[25]

All 1664 compounds were screened at 10 μM, resulting in the identification of three inhibitors targeting Faah as well as an as-yet unidentified serine hydrolase (Fig. 2B–C). The Faah inhibitors **4–6** all contain a urea moiety, a functional group that is frequently present in clinically evaluated Faah inhibitors.[14,26] This preference is likely related to the structural similarity between ureas and the natural amide substrates of Faah. All identified inhibitors display potencies in the micromolar range and possess favourable physicochemical properties (Fig. 2C–E).

2.2 *Inhibition Profile of Inhibitor 7*

Interestingly, epoxide **7** inhibited an unidentified serine hydrolase. To identify the target of **7** chemical proteomics was performed on both the membrane and cytosolic fractions of mouse brain proteome, as described previously.[27] Briefly, lysates were treated with either dimethyl sulfoxide (DMSO) or 10 μM of compound **7**, followed by labelling with FP-biotin and THL-biotin. Biotinylated proteins were enriched by streptavidin affinity purification and subsequently digested with trypsin. The resulting peptides were analysed by liquid chromatography-mass spectrometry (LC-MS).

Abhd2 was identified as the only protein that was significantly inhibited by **7** (Fig. 3A-B). To validate this interaction, human FLAG-tagged ABHD2 was overexpressed in U2OS cells, as described previously.[28] After cell lysis, lysates were treated with either **7** or DMSO and subsequently labelled with probe **3**. Consistent with the proteomics data, compound **7** inhibited ABHD2 with a micromolar potency (Fig. 3C). Notably, the apparent molecular weight of the unidentified protein band observed in the initial gel-based screen (approximately 80–100 kDa) does not correspond to Abhd2 (48 kDa). This suggests that additional targets of compound **7** remain to be identified.

3. Conclusion

We established a multiplexed gel-based ABPP platform that combines three competitive experiments into a single SDS-PAGE

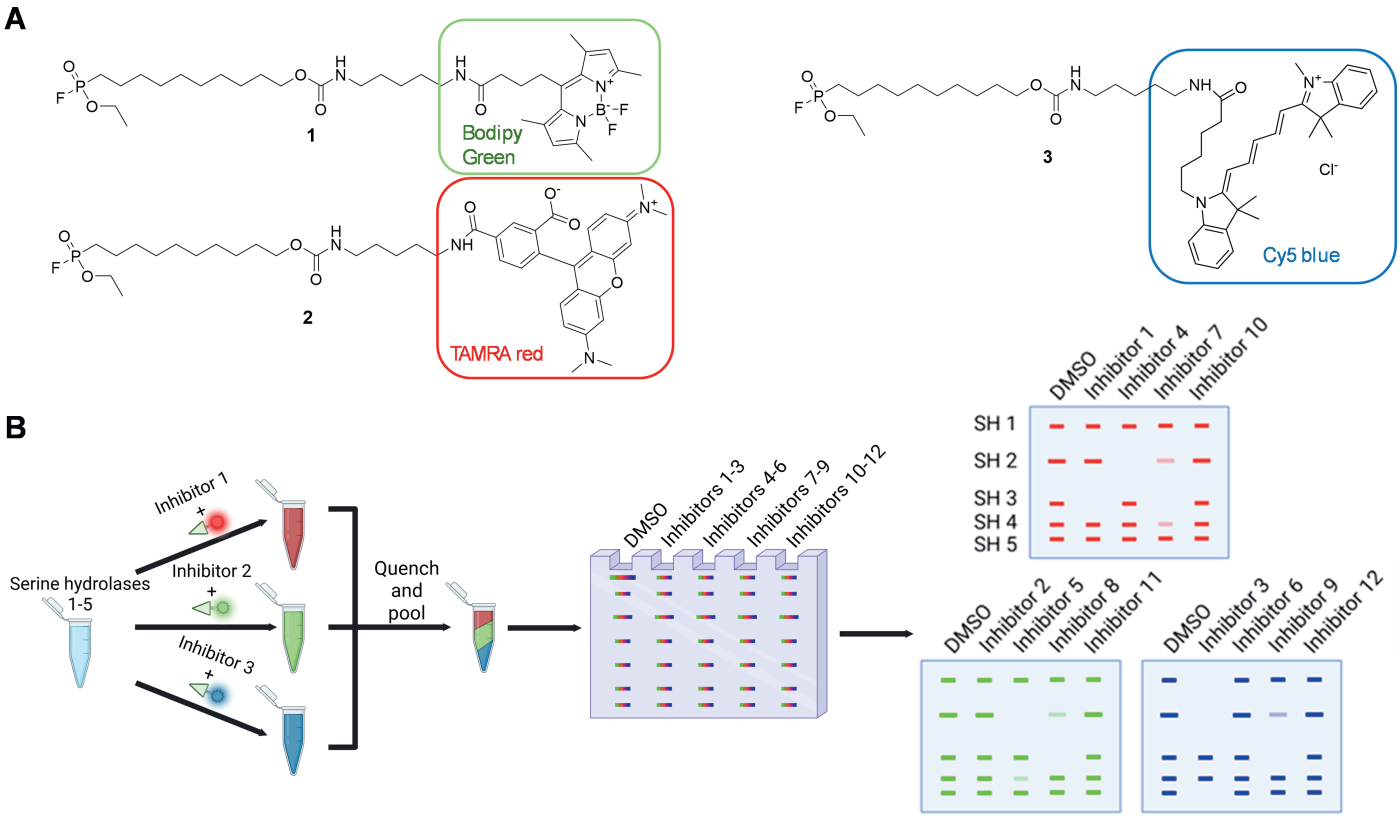

Fig. 1. **Activity-based probes and schematic overview of the gel-based multiplexed ABPP assay**. A) Previously developed Bodipy-FL (green) (**1**) and TAMRA (red) (**2**) fluorophosphonate probes, and Cy5 (blue) probe (**3**) synthesised in this work. B) Schematic overview of the serine hydrolase-focused compound library screen using a gel-based multiplexed ABPP assay. Mouse brain proteome was treated with three different inhibitors and subsequently with the three distinct fluorescent probes after which the quenched competition experiments were combined and resolved in a single SDS-PAGE lane, increasing the number of inhibitors that can be interrogated on one SDS-PAGE gel.

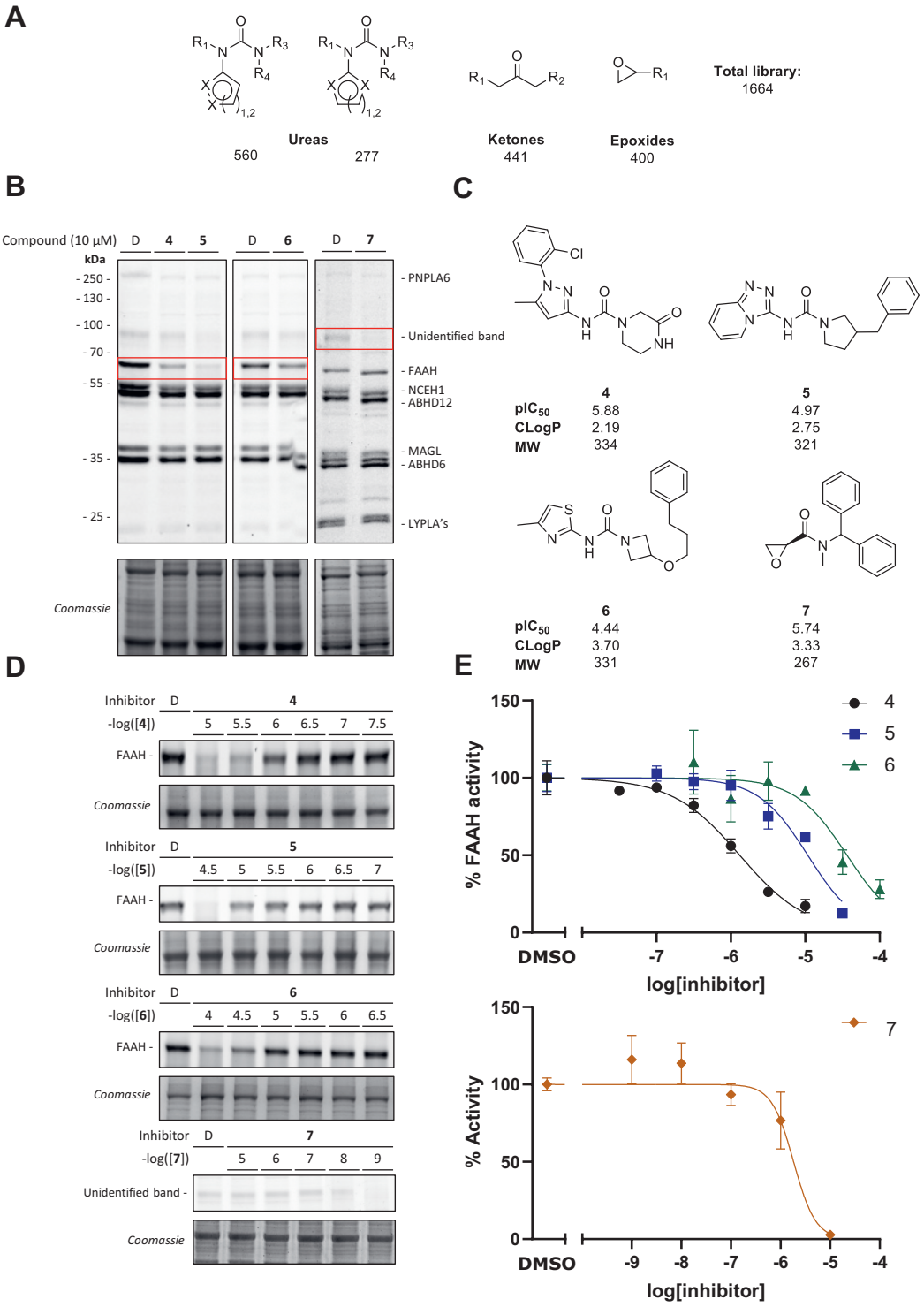

Fig. 2. **Identification of novel serine hydrolase inhibitors using gel-based ABPP in mouse brain proteome**. A) Overview of the electrophiles present in the compound library selected from Enamine. Urea compounds were filtered for molecules featuring an aromatic heterocycle directly adjacent to the urea moiety. These aromatic heterocycles had to contain at least two heteroatoms in the vicinity of the urea moiety. B) Mouse brain proteome was incubated with compounds **4-7** or DMSO and subsequently with probe **3**. The competition experiments were quenched with Laemmli buffer and resolved on SDS-PAGE. Ureas **4-6** inhibit Faah, while epoxide **7** inhibits an unidentified band. C) Structural, biochemical, and physicochemical data of the novel inhibitors **4-7**. D-E) Dose-response curves of the inhibitors **4-7** (n=3) against their respective targets, including a representative gel.

lane using fluorophosphonate probes bearing distinct fluorophores. This approach markedly improves throughput while preserving target resolution. We applied this multiplexed assay to screen a curated set of 1664 compounds from a Serine Hydrolase-focused Enamine library in mouse brain proteome. This target-agnostic screen yielded 3 micromolar Faah inhibitors **4–6** bearing urea warheads and identified epoxide **7** as an inhibitor of Abhd2.

Faah has received a lot of attention as a therapeutic target for a wide range of disorders. This has led to the development of the nanomolar potent Faah inhibitor PF04457845, which was well-tolerated in healthy volunteers, but has currently not yet been approved for therapeutic applications.[29,30] Target deconvolution of compound **7** by chemical proteomics identified Abhd2 as a target. Abhd2 is implicated in several physiological processes, including

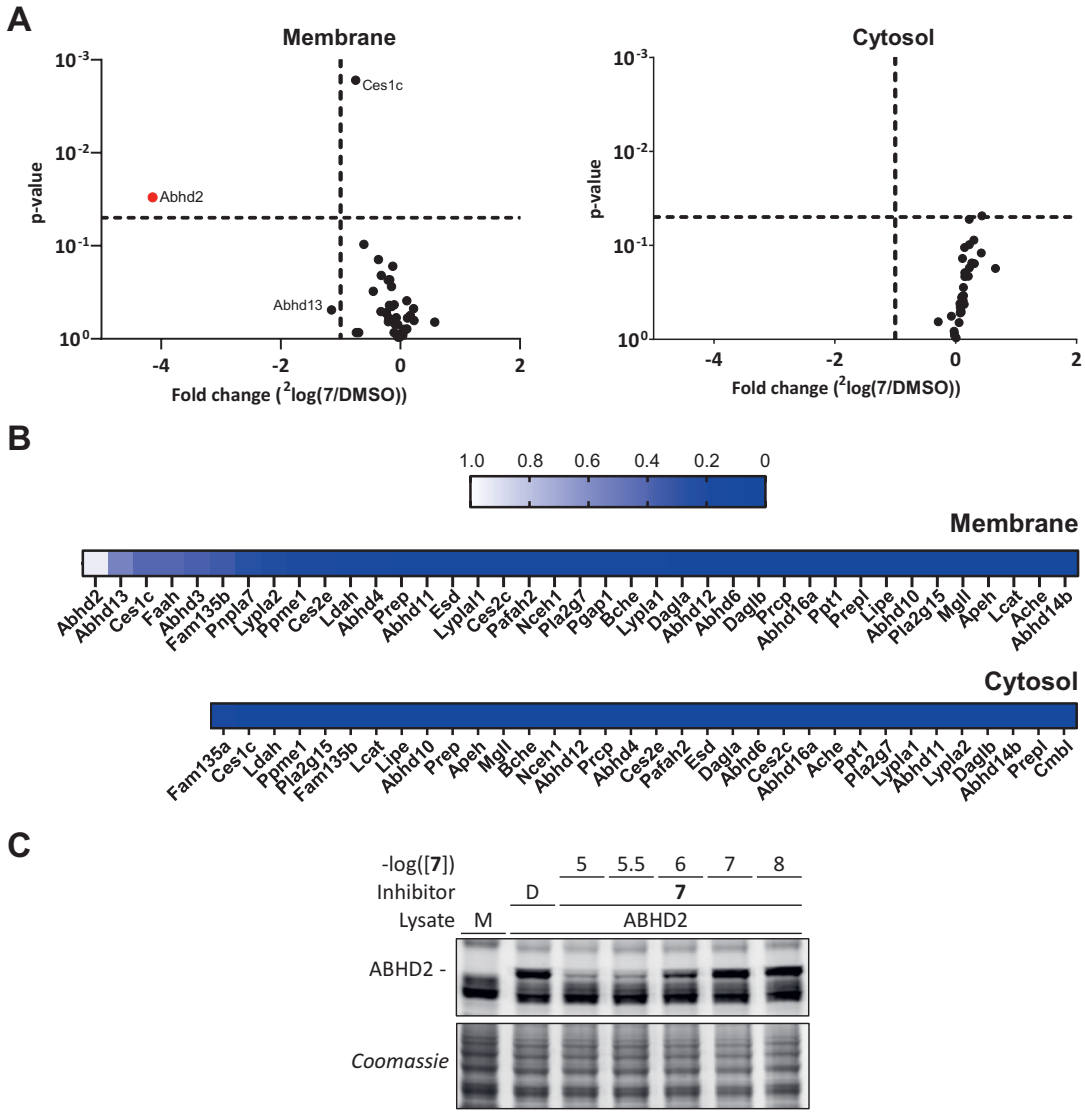

Fig. 3. Target identification of epoxide 7**. A)** Volcano plot showing only Abhd2 was significantly inhibited by **7** at 10 μM in membrane fractions of mouse brain lysate as analysed by MS-based proteomics. The proteomic data are visualized in volcano plots where the x-axis shows the fold change of proteins present in the inhibitor-treated *vs* the DMSO-treated lysates and the y-axis shows the p-value. B) Heat map of the proteomics data depicted in A). C**)** Dose-response assay of **7** against Abhd2 in lysate of U2OS cells overexpressing Abhd2.

sperm maturation. We have previously identified another class of ABHD2 inhibitors.[28,31]

Despite its strengths, the approach presented here has several limitations. Currently, serine proteases remain poorly detected using fluorophosphonate probes, which biases the platform toward metabolic SHs. The resolution of the gel-based assay is also limited, therefore only highly abundant serine hydrolases are detected. In addition, weak non-covalent interactions may be missed, as the covalent activity-based probes can outcompete low-affinity binders.

Overall, this work delivers a scalable workflow that couples rapid, gel-based activity profiling with orthogonal chemoproteomic target identification, thereby converting covalent library hits into annotated chemical matter. The identification of Faah inhibitors and an Abhd2 inhibitor illustrates how this approach can facilitate the discovery of selective hits and enable mechanism-of-action studies on underexplored proteins. Future efforts should expand target coverage to additional mSHs, integrate quantitative MS-based ABPP at earlier stages to generate highly annotated selectivity maps, and develop structure-guided analogues of the identified hits to improve potency and cellular activity.

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Author Contributions

F. H. G. t. B. designed and performed the experiments, analysed the results and wrote the paper. P. R. and M. H. performed the experiments and analysed the results. M. v. d. S. and A. P. A. J. designed the experiments and wrote the paper.

Conflict of interest

A. P. A. Janssen is a co-founder of Omivera B. V. The remaining authors have no conflicts of interest to declare.

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[1] D. A. Bachovchin, B. F. Cravatt, *Nat. Rev. Drug Discov*ery **2012**, *11*, 52, https://doi.org/10.1038/nrd3620.
[2] J. Z. Long, B. F. Cravatt, *Chem. Rev*. **2011**, *111*, 6022, https://doi.org/10.1021/cr200075y.

[3] A. Rajendran, K. Vaidya, J. Mendoza, J. Bridwell-Rabb, S. S. Kamat, *Biochem.* **2020**, *59*, 183, https://doi.org/10.1021/acs.biochem.9b00703.
[4] A. M. van Bennekum, E. A. Fisher, W. S. Blaner, E. H. Harrison, *Biochem.* **2000**, *39*, 4900, https://doi.org/10.1021/bi9927235.
[5] A. Roussel, S. Canaan, M.-P. Egloff, M. Rivière, L. Dupuis, R. Verger, C. Cambillau, *J. Biol. Chem*. **1999**, *274*, 16995, https://doi.org/10.1074/jbc.274.24.16995.
[6] E. Giacobini, *Neurochem. Res*. **2003**, *28*, 515, https://doi.org/10.1023/A:1022869222652.
[7] N. van Egmond, V. M. Straub, M. van der Stelt, *Annu. Rev. Pharmacol. Toxicol*. **2021**, *61*, 441, https://doi.org/10.1146/annurev-pharmtox-030220-112741.
[8] B. Sternby, D. Hartmann, B. Borgström, Å. Nilsson, *Clin. Nutr.* **2002**, *21*, 395, https://doi.org/10.1054/clnu.2002.0565.
[9] M. Weinstock, *CNS Drugs* **1999**, *12*, 307, https://doi.org/10.2165/00023210-199912040-00005.
[10] J. Doupis, A. Veves, *Adv. Ther*. **2008**, *25*, 627, https://doi.org/10.1007/s12325-008-0076-1.
[11] J. R. White, *Clin. Diabetes* **2008**, *26*, 53, https://doi.org/10.2337/diaclin.26.2.53.
[12] D. A. Bachovchin, T. Ji, W. Li, G. M. Simon, J. L. Blankman, A. Adibekian, H. Hoover, S. Niessen, B. F. Cravatt, *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 20941, https://doi.org/10.1073/pnas.1011663107.
[13] D. K. Nomura, M. M. Dix, B. F. Cravatt, *Nat. Rev. Cancer* **2010**, *10*, 630, https://doi.org/10.1038/nrc2901.
[14] D. S. Johnson, C. Stiff, S. E. Lazerwith, S. R. Kesten, L. K. Fay, M. Morris, D. Beidler, M. B. Liimatta, S. E. Smith, D. T. Dudley, N. Sadagopan, S. N. Bhattachar, S. J. Kesten, T. K. Nomanbhoy, B. F. Cravatt, K. Ahn, *ACS Med. Chem. Lett*. **2011**, *2*, 91, https://doi.org/10.1021/ml100190t.
[15] A. E. Weber, *J. Med. Chem*. **2004**, *47*, 4135, https://doi.org/10.1021/jm030628v.
[16] M. Jiang, M. C. W. Huizenga, J. L. Wirt, J. Paloczi, A. Amedi, R. J. B. H. N. van den Berg, J. Benz, L. Collin, H. Deng, X. Di, W. F. Driever, B. I. Florea, U. Grether, A. P. A. Janssen, T. Hankemeier, L. H. Heitman, T. W. Lam, F. Mohr, A. Pavlovic, I. Ruf, H. van den Hurk, A. F. Stevens, D. van der Vliet, T. van der Wel, M. B. Wittwer, C. A. A. van Boeckel, P. Pacher, A. G. Hohmann, M. van der Stelt, *Nat. Commun*. **2023**, *14*, 1, https://doi.org/10.1038/s41467-023-43606-3.
[17] J. Z. Long, W. Li, L. Booker, J. J. Burston, S. G. Kinsey, J. E. Schlosburg, F. J. Pavón, A. M. Serrano, D. E. Selley, L. H. Parsons, A. H. Lichtman, B. F. Cravatt, *Nat. Chem. Biol*. **2009**, *5*, 37, https://doi.org/10.1038/nchembio.129.
[18] J. S. Cisar, O. D. Weber, J. R. Clapper, J. L. Blankman, C. L. Henry, G. M. Simon, J. P. Alexander, T. K. Jones, R. A. B. Ezekowitz, G. P. O'Neill, C. A. Grice, *J. Med. Chem*. **2018**, *61*, 9062, https://doi.org/10.1021/acs.jmedchem.8b00951.
[19] N. Zhu, A. P. A. Janssen, M. van der Stelt, *Isr. J. Chem*. **2023**, *63*, e202200115, https://doi.org/10.1002/ijch.202200115.
[20] A. P. A. Janssen, D. van der Vliet, A. T. Bakker, M. Jiang, S. H. Grimm, G. Campiani, S. Butini, M. van der Stelt, *ACS Chem. Biol.* **2018**, *13*, 2406, https://doi.org/10.1021/acschembio.8b00534.
[21] J. C. Powers, J. L. Asgian, Ö. D. Ekici, K. E. James, *Chem. Rev*. **2002**, *102*, 4639, https://doi.org/10.1021/cr010182v.
[22] M. Gehringer, S. A. Laufer, *J. Med. Chem*. **2019**, *62*, 5673, https://doi.org/10.1021/acs.jmedchem.8b01153.
[23] A. Albeck, *Drug Dev. Res*. **2000**, *50*, 425, https://doi.org/10.1002/1098-2299(200007/08)50:3/4%3C425::AID-DDR25%3E3.0.CO;2-B.
[24] P. R. A. Zanon, F. Yu, P. Z. Musacchio, L. Lewald, M. Zollo, K. Krauskopf, D. Mrdović, P. Raunft, T. E. Maher, M. Cigler, C. J. Chang, K. Lang, F. D. Toste, A. I. Nesvizhskii, S. M. Hacker, *Nat. Chem.* **2025**, *17*, 1712, https://doi.org/10.1038/s41557-025-01902-z.
[25] T. M. Gloster, R. Madsen, G. J. Davies, *Org. Biomol. Chem*. **2007**, *5*, 444, https://doi.org/10.1039/B616590G.
[26] J. M. Keith, W. M. Jones, M. Tichenor, J. Liu, M. Seierstad, J. A. Palmer, M. Webb, M. Karbarz, B. P. Scott, S. J. Wilson, L. Luo, M. L. Wennerholm, L. Chang, M. Rizzolio, R. Rynberg, S. R. Chaplan, J. G. Breitenbucher, *ACS Med. Chem. Lett.* **2015**, *6*, 1204, https://doi.org/10.1021/acsmedchemlett.5b00353.
[27] E. J. Van Rooden, B. I. Florea, H. Deng, M. P. Baggelaar, A. C. M. Van Esbroeck, J. Zhou, H. S. Overkleeft, M. Van Der Stelt, *Nat. Protoc*. **2018**, *13*, 752, https://doi.org/10.1038/nprot.2017.159.
[28] M. P. Baggelaar, H. den Dulk, B. I. Florea, D. Fazio, N. Bernabò, M. Raspa, A. P. A. Janssen, F. Scavizzi, B. Barboni, H. S. Overkleeft, M. Maccarrone, M. van der Stelt, *ACS Chem. Biol*. **2019**, *14*, acschembio.9b00640, https://doi.org/10.1021/acschembio.9b00640.
[29] J. P. Huggins, T. S. Smart, S. Langman, L. Taylor, T. Young, *Pain* **2012**, *153*, 1837, https://doi.org/10.1016/j.pain.2012.04.020.
[30] G. L. Li, H. Winter, R. Arends, G. W. Jay, V. Le, T. Young, J. P. Huggins, *Br. J. Clin. Pharmacol*. **2012**, *73*, 706, https://doi.org/10.1111/j.1365-2125.2011.04137.x.
[31] G. Bononi, T. Tuccinardi, F. Rizzolio, C. Granchi, *J. Med. Chem*. **2021**, *64*, 9759, https://doi.org/10.1021/acs.jmedchem.1c00624.

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