HHS Public Access

Author manuscript

*Chem Commun (Camb)*. Author manuscript; available in PMC 2024 July 06.

Published in final edited form as:

*Chem Commun (Camb)*. ; 59(55): 8552–8555. doi:10.1039/d3cc02540c.

# Firefly luciferin methyl ester illuminates the activity of multiple serine hydrolases

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## Abstract

Firefly luciferin methyl ester is hydrolyzed by monoacylglycerol lipase MAGL, amidase FAAH, poorly-characterized hydrolase ABHD11, and hydrolases known for S-depalmitoylation (LYPLA1/2), not just esterase CES1. This enables activity-based bioluminescent assays for serine hydrolases and suggests that the 'esterase activity' responsible for hydrolyzing ester prodrugs is more diverse than previously supposed.

Fireflies are beetles that emit a green glow when the enzyme firefly luciferase adenylates and oxidizes the small molecule D-luciferin. The chemistry of bioluminescence requires a free carboxylic acid, and beetle luciferases evolved from enzymes that adenylate fatty acids (Figure 1).[1–3] Embracing D-luciferin and analogues as fatty-acid mimics previously led us to design luciferin amides as probes that specifically report on the enzymatic activity of fatty acid amide hydrolase (FAAH),[4] a membrane-bound enzyme that hydrolyzes fatty acid amide neurotransmitters such as anandamide and oleamide, and has been a popular target for pharmaceutical development.[5,6] Hydrolysis of luciferin amides in the presence of firefly luciferase results in bioluminescence, enabling detection of FAAH activity in live cells and in vivo.[4,7–9] Building on this concept, we hypothesized that luciferin esters could similarly be substrates for lipases that regulate signaling lipids, in particular monoacylglycerol lipase (MAGL, aka MGLL) and the alpha/beta hydrolase domain (ABHD) enzymes ABHD6 and ABHD12, as these enzymes all hydrolyze the endocannabinoid 2-arachidonoyl glycerol (2-AG) to arachidonic acid (Figure 1).[10]

Esters of D-luciferin have long been known to be cell-permeable and hydrolyzed by "esterase activity",[11,12] but the specific enzyme(s) responsible for ester hydrolysis have not been well described. It has been reported that D-luciferin methyl ester is specifically hydrolyzed by human carboxyesterase 1 (CES1).[13] However, we have found that ethyl esters of D-luciferin and the luciferin analogue CycLuc1 can be hydrolyzed by the amidase FAAH,[4] and many mammalian cells do not appreciably express CES1 or FAAH, yet still hydrolyze esters.[14,15] Here we show that D-luciferin methyl ester is a substrate for a number of serine hydrolases, including MAGL, lysophosphoplipase 1 (LYPLA1), lysophospholipase 2 (LYPLA2), and

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ABHD11.[16] Activity-based bioluminescence assays[17] for these hydrolases are thus enabled, complementing activity-based protein profiling (ABPP) methods,[18] and adding the ability to study serine hydrolases in live cells. More broadly, these results also suggest that the scope of enzymes capable of hydrolyzing ester prodrugs extends beyond the canonical carboxyesterase family,[19,20] with implications for their selective unmasking in different cell types.

Human PC3 prostate and U87 glioma cell lines both express MAGL and ABHD6, but not CES1 or FAAH.[14,15,21,22] We therefore transfected PC3 and U87 cells with firefly luciferase, then treated them with 10 μM D-luciferin methyl ester. A strong bioluminescent signal was observed from both cell lines, exceeding that of 10 μM D-luciferin itself (Figure S1),[11] indicating that hydrolysis of the ester to the free carboxylate had occurred (Figure 2). We observed a significant and dose- dependent reduction in bioluminescence from cells pre-incubated with the MAGL inhibitors JZL184 and KML29,[23] suggesting that MAGL contributes to this hydrolytic activity. Treatment of cells with the nonspecific serine protease inhibitor MAFP resulted in an even greater reduction in bioluminescence than KML29, suggesting that, in addition to MAGL, other cellular hydrolases could also cleave luciferin esters. However, the ABHD6 inhibitor[24] KT182 did not inhibit bioluminescence, indicating that ABHD6 was not responsible, and the CES1 inhibitor[25] WWL113 similarly had no effect, confirming the lack of CES1 involvement in these cells.

To better tease apart which enzymes are capable of hydrolyzing D-luciferin methyl ester, we expressed a panel of candidate hydrolases in HEK293 cells. We focused on human serine hydrolases that have demonstrated activity toward fatty acyl glycerols (MAGL, ABHD6, ABHD12, FAAH, CES1),[10] but also included several serine hydrolases that are less well characterized. LYPLA1 and LYPLA2,[26] also known as acyl-protein thioesterase (APT) 1 and 2, are perhaps best known for hydrolyzing palmitate thioesters from proteins.[27,28] The reported ability of LYPLA2 to hydrolyze prostaglandin glycerol esters[29] suggested to us that it may also be capable of hydrolyzing luciferin esters. In addition, we included ABHD11, a poorly-characterized serine hydrolase that is presumed to act on lipids, but whose endogenous substrate(s) have not been firmly established.[30–34] As controls, we expressed the corresponding mutant enzymes in which the key catalytic serine residue was mutated to alanine. When co-expressed with firefly luciferase, a number of these hydrolytic enzymes demonstrated an ability to enhance bioluminescence from D-luciferin methyl ester compared to their control (Figure 3, Figure S2). Active hydrolase expression had no effect on bioluminescence from D-luciferin (Figure S3). These results confirmed that, in addition to CES1 and FAAH,[4,13] the hydrolytic activity of MAGL can cleave D-luciferin methyl ester. Moreover, LYPLA1, LYPLA2, and, unexpectedly, ABHD11, were also found to greatly increase the bioluminescent signal. We expect that this list is not exhaustive. On the other hand, the monoacylglycerol lipases ABHD6 and ABHD12 surprisingly showed no activity toward D-luciferin methyl ester (Figure 3) despite their known activity toward 2-AG (Figure 1).[10] It is possible that active ABHD6 and ABHD12 are poorly expressed in our assay, but the previous successful expression of these enzymes suggests that this is not the case.[35,36] Moreover, the ABHD6 results are consistent with the inability of KT182 to block bioluminescence from D-luciferin methyl ester in PC3 and U87 cells (Figure 2).

We next asked whether D-luciferin methyl ester could function as an activity-based bioluminescence probe for detecting the inhibition of serine hydrolases overexpressed in HEK293 cells without interference from endogenous hydrolase activity. ML226 has been reported to be a specific inhibitor of ABHD11.[37] We found that ML226 potently inhibits the bioluminescent signal from D-luciferin methyl ester in cells expressing ABHD11 in a dose-dependent manner, bringing the bioluminescence down to the level of the inactive S141A ABHD11 control, whereas the MAGL inhibitors KML29 and JZL184 have no effect (Figure 4). This suggests that D-luciferin methyl ester can be used to identify ABHD11 inhibitors using this live cell assay, and that this general strategy has potential for the identification and characterization of inhibitors of other serine hydrolases.

Our results indicate that D-luciferin methyl ester is not just a substrate for CES1 and suggests that luciferin esters in general hold promise as probes for the detection of numerous serine hydrolases, particularly those with lipid substrates. D-Luciferin methyl ester is not as specific for lipases as D-luciferin and CycLuc1 amides are for FAAH, and a challenge for the future translation of luciferin esters to in vivo imaging applications is the promiscuous activity of CES1. Nonetheless, D-luciferin methyl ester can presently be used to selectively detect the endogenous activity of MAGL in live cells. Furthermore, by expressing active and inactive forms of hydrolytic enzymes, the activity from other lipases such as ABHD11 can be detected, enabling the identification and characterization of inhibitors in situ. Many serine hydrolases are membrane-bound and remain poorly characterized,[16,38] and even those that have been the subject of study continue to reveal new surprises.[39] New tools to identify and validate the selectivity and specificity[40] of inhibitors in live cells are thus valuable. Given the ever-expanding scope of luminogenic luciferin analogues,[8,41–44] we anticipate that modified luciferin structures as well as the incorporation of different esters can be utilized in combination to further refine the specificity and expand the scope of activity-based bioluminescence probes for enzymes that process lipid substrates, potentially including ABHD6 and ABHD12, among others. Finally, our results indicate that the hydrolytic activity typically ascribed to “esterases” can also arise from several serine hydrolases that are involved in specialized aspects of lipid metabolism. This includes, but is not limited to, FAAH, MAGL, LYPLA1, LYPLA2, and ABHD11. Notably, the use of FAAH for amide prodrug activation has recently been reported to extend beyond luciferin analogues.[45] The activity of serine hydrolases like MAGL toward ester prodrugs may similarly have implications for their subsequent unmasking in different cell types.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This work was supported by the U.S. National Institutes of Health (DA039961 and EB013270). I.M. performed synthesis and HEK cell assays; K.L.L. performed PC3 and U87 cell assays; S.C.M. conceived the project, directed research, and wrote the paper with input from I.M.

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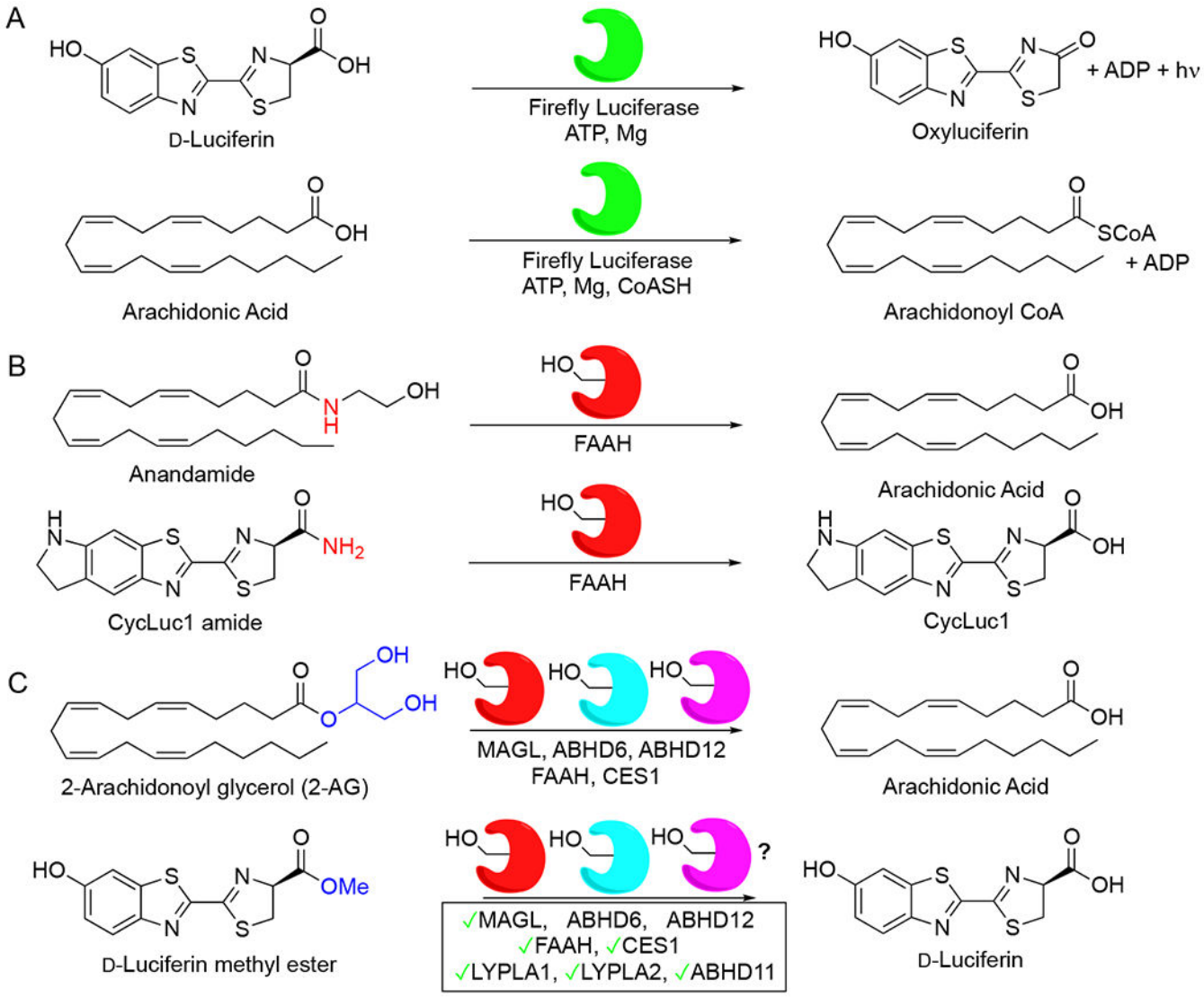


**Figure 1.**

Building bioluminescent sensors for serine hydrolases. A) Firefly luciferase oxidizes D-luciferin to emit light, but also has fatty acyl-CoA synthetase activity; B) In previous work, we embraced D-luciferin and luciferin analogues as fatty acid mimics, finding that luciferin amides can be hydrolyzed by FAAH, forming the basis for bioluminescent detection of FAAH activity; C) Here, we propose that D-luciferin methyl ester can similarly serve as a sensor of monoacylglycerol lipase activity.

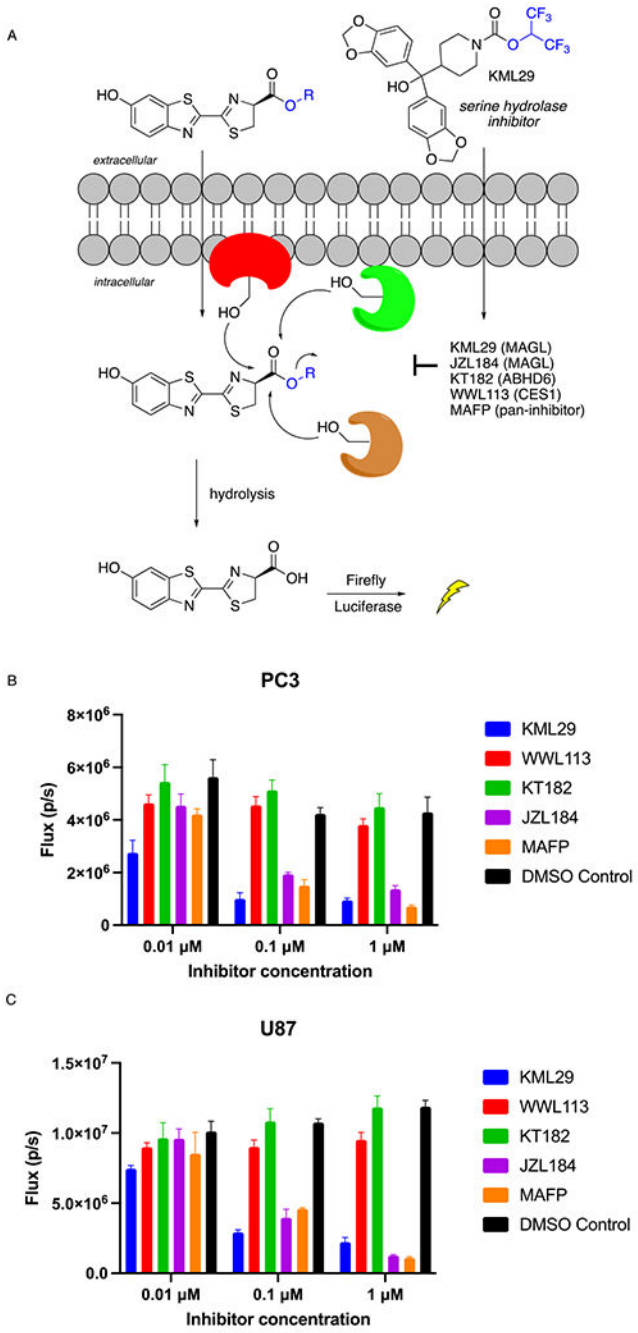


**Figure 2.**

Bioluminescence from luciferase-transfected cells after D-luciferin methyl ester treatment. A) Hydrolysis of the methyl ester generates D-luciferin, resulting in bioluminescence. Inhibition of the enzyme(s) responsible for hydrolysis inhibits the bioluminescent signal; B,C) Luciferase-expressing PC3 prostate cells (B) and U87 glioma cells (C) were treated with the indicated serine hydrolase inhibitors, then imaged with D-luciferin methyl ester (10 μM).

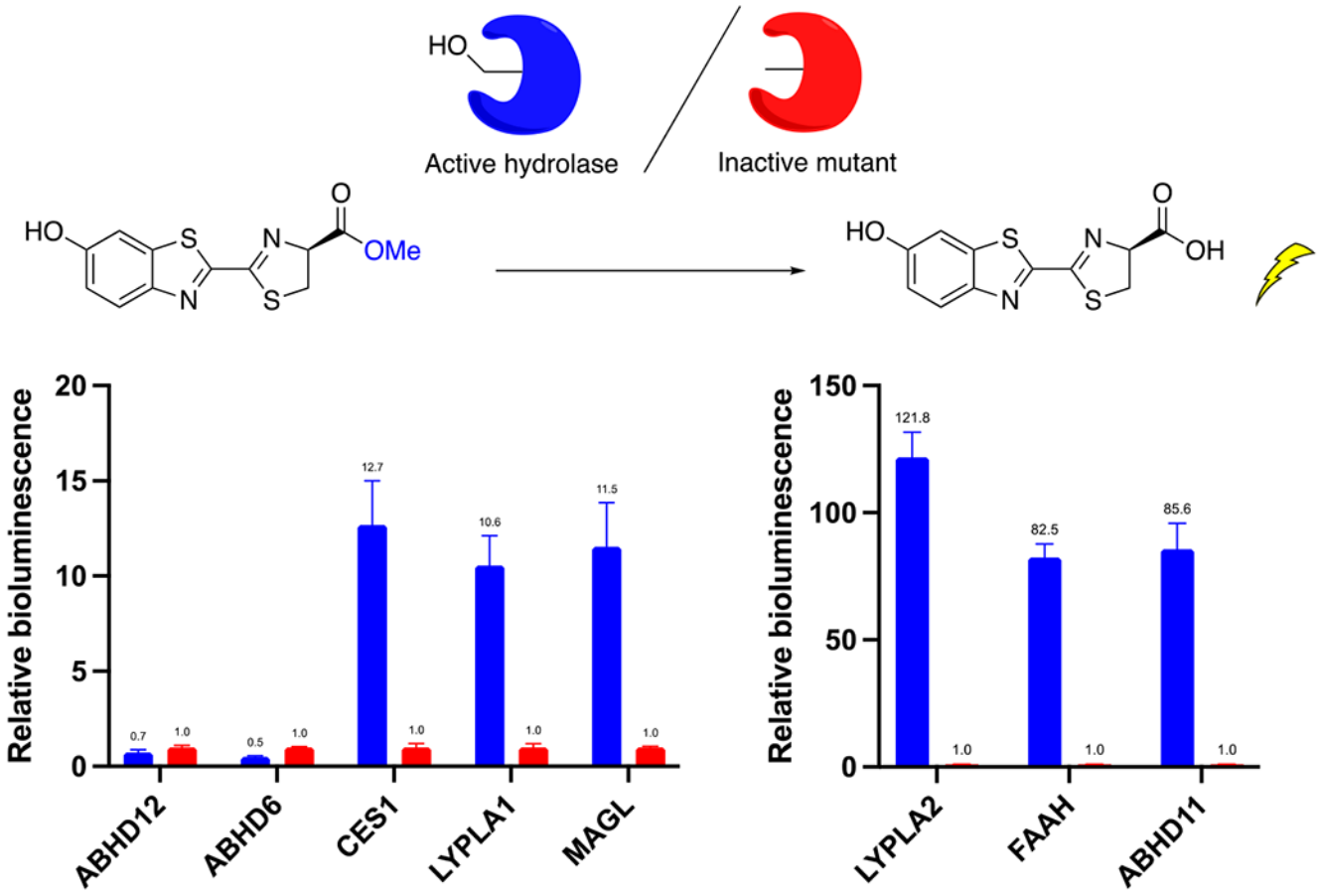


**Figure 3.**

Effect of serine hydrolase expression on bioluminescence with D-luciferin methyl ester in HEK293 cells. Represented as the fold-increase in bioluminescence for the active serine hydrolase compared to its control inactive serine to alanine mutant.

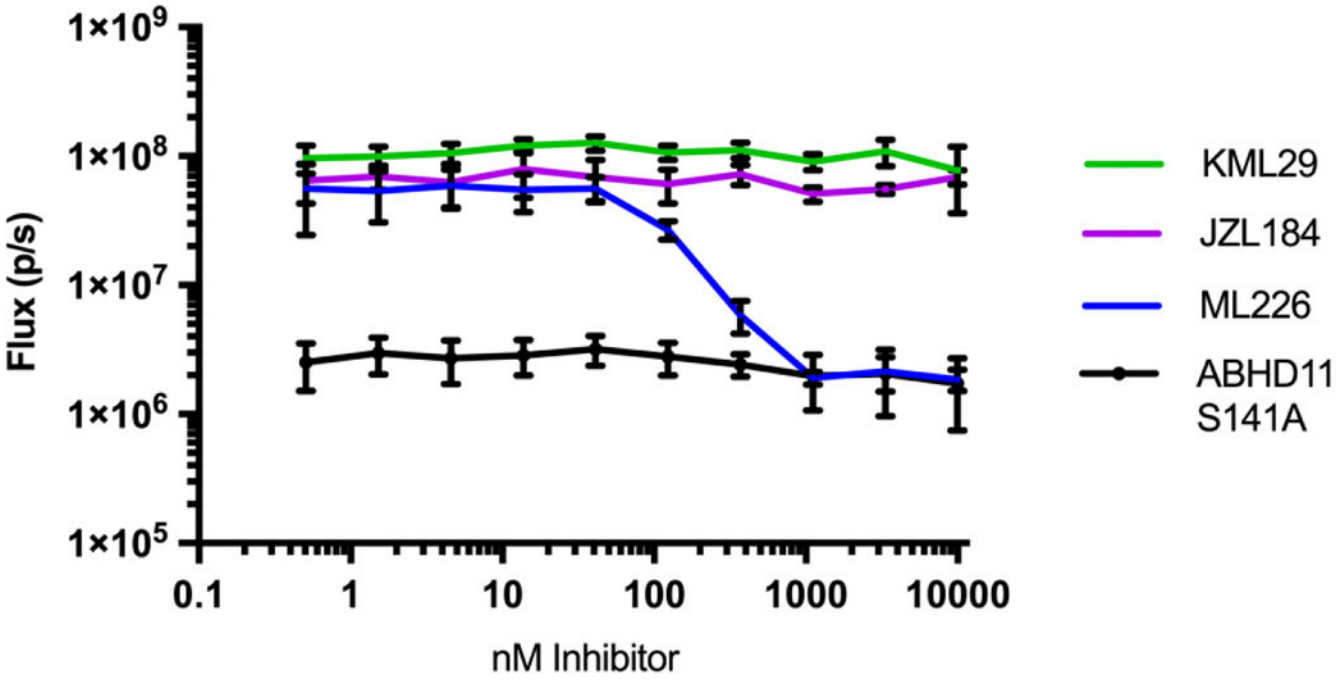


**Figure 4.**

Bioluminescence emission from HEK293 cells expressing ABHD11 and firefly luciferase treated with D-luciferin methyl ester and the indicated serine hydrolase inhibitors, compared to the inactive ABHD11-S141A mutant control.