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Luciferins Behave Like Drugs

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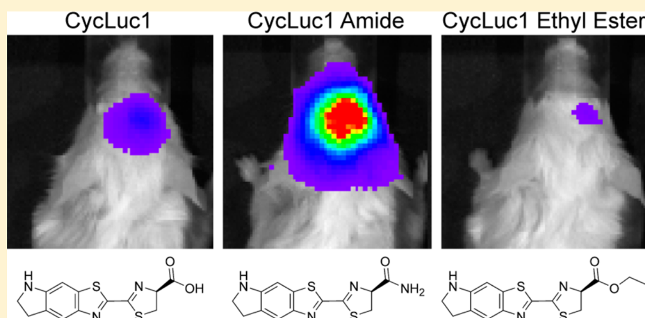
ABSTRACT: The light emission chemistry of firefly luciferase can be harnessed to reveal otherwise invisible biological processes occurring in the brains of live animals. Though powerful, the need for the luciferase substrate D-luciferin to traverse the blood-brain barrier poses limitations on the sensitivity and interpretation of these experiments. In this Viewpoint, we discuss bioluminescent imaging probes for the enzyme fatty acid amide hydrolase (FAAH) and the broader implications for optical imaging and drug delivery in the brain.



KEYWORDS: *blood-brain barrier, FAAH, bioluminescence imaging, drug delivery, luciferase, luciferin*

Luciferins do not treat disease, but in many ways these exogenous molecules act like drugs. Instead of a eliciting a therapeutic response, luciferins emit light when they encounter their target (luciferase). If you want to light up the brain in a luciferase-expressing mouse, you essentially have a drug delivery problem: how to get across the blood-brain barrier. Generally, this means you want a molecule that is small (<500 MW), lipophilic enough to readily diffuse across cell membranes, and preferably not a substrate for the armada of efflux pumps poised at the border, ready to send your molecule packing. D-Luciferin, the substrate for firefly luciferase, gets a gold star for small size, but everything else has room for improvement.

Fireflies produce light by the oxidation of D-luciferin to an excited-state molecule (Figure 1). Recent work has shown that the enzyme firefly luciferase will accept many substrate analogues, allowing tuning of the molecular properties of the luciferin.[1,2] These synthetic luciferins are not necessarily "better" than the natural substrate. Indeed, if firefly luciferase is provided with a saturating concentration of luciferin in a test tube, the rate of photon emission is higher with the natural substrate D-luciferin than with any synthetic luciferin analogue yet made.[1] However, in vivo, and particularly in the brain, the access of D-luciferin is limited. Saturating levels of D-luciferin are not achieved after intraperitoneal (IP) injection into live mice.[3] Under these conditions, luciferin analogues with higher cell permeability and lower K_m values for luciferase can perform better than the natural substrate, and do so at substantially lower imaging doses.[1,2]

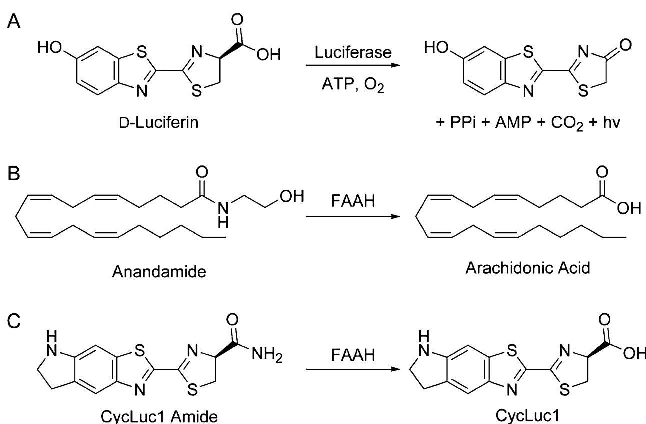


Figure 1. (A) Firefly luciferase oxidizes its substrate D-luciferin to emit light. This chemistry requires a free carboxylate. (B) FAAH hydrolyzes anandamide and many other fatty acid amides. (C) Luciferin amides can be hydrolyzed by FAAH to unmask substrates for luciferase (ref 2).

A complete picture of all the factors that restrict D-luciferin access to the brain has not yet emerged. In general, small lipophilic compounds can penetrate the blood-brain barrier (BBB), while large and/or hydrophilic compounds are excluded. D-Luciferin is small (<300 MW), but relatively polar. Its ability to access the brain by simple diffusion is therefore expected to be modest. Furthermore, there are many efflux pumps (aka "drug resistance pumps") that actively remove molecules from the brain. These include ABCG2, Pgp (ABCB1), MRP1 (ABCC1), and MRP4 (ABCC4).[1,4] D-Luciferin was thought to be a substrate only for ABCG2.[1,4] However, it has recently been reported to be a substrate for MRP4 as well.[4] The relevance of this finding for access to the brain has not yet been established.

It is important to be mindful of the role that the blood-brain barrier plays in restricting the access of the luciferin substrate. Some experimental techniques or disease states can disrupt the BBB. For example, injection of luciferase-expressing cells into

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the brain will disrupt the BBB, and immediate imaging of those injected cells is therefore unlikely to reflect an intact BBB.[3] And as recently demonstrated by Ayzenberg et al.,[5] neuroinflammation also has the potential to disrupt the BBB. The bioluminescent signal from D-luciferin may increase simply due to improved substrate access to brain tissue, and be confused with an increase in gene expression.[5] Similarly, the growth of brain tumors that disrupt the BBB may also affect the interpretation of the resulting imaging data. Potentially, the use of luciferins that are less sensitive to BBB disruption could help avoid these issues.

Synthetic luciferin substrates with higher lipophilicity and lower K_m values for luciferase can improve imaging in the brain (Figure 2).[1,2] These substrates are expected to more readily diffuse across cell membranes, and due to their lower K_m values less substrate is needed to saturate the luciferase. Changes to the luciferin structure may also affect efflux pump activity, but it is not yet clear if or how the efflux of synthetic luciferins from the brain differs from D-luciferin.

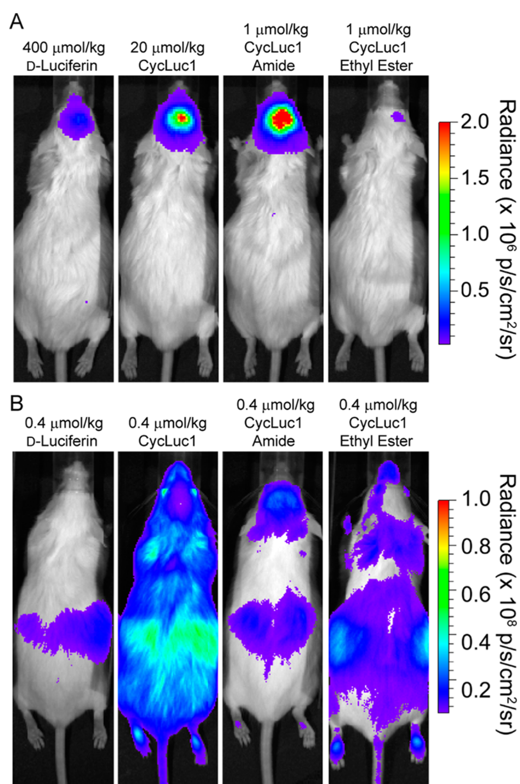

Figure 2. Bioluminescence imaging in luciferase-expressing mice (Adapted with permission from ref 2. Copyright 2015 American Chemical Society). (A) Adeno-associated virus (AAV)9 was used to express luciferase only in the striatum of the brain. Photon flux from the same mouse was compared using the indicated luciferin analogue and dose (i.p. injection). (B) The same set of luciferin analogues was compared at equal dose in a trangenic mouse that ubiquitously expresses luciferase.

All substrates for firefly luciferase require a free carboxylate. At physiological pH, the presence of an ionized carboxylate on the luciferin is thus another factor that limits its cell permeability and ability to cross the blood-brain barrier. This is where things get really interesting. Fatty acid amide hydrolase (FAAH) is the enzyme responsible for the hydrolysis of anandamide, an endogenous ligand for the cannabinoid receptor CB1 (Figure 1). We synthesized luciferin amides designed to be substrates for FAAH, a promising drug target for the treatment of pain and anxiety.[2] Cleavage of luciferin amides by FAAH liberates a luciferin substrate, allowing sensitive and specific bioluminescence detection of FAAH activity in the brain and periphery.[2] This was all by design—but what was surprising was that luciferin amides improve brain bioluminescence in live mice over both D-luciferin and their parent luciferins (Figure 2).[2] Far less substrate is needed for imaging, and the photon flux is increased. Luciferin amides are acting as luciferin "pro-drugs".

When imaging in mice that express luciferase in all tissues, bioluminescence is dominated by superficial tissues—wherever the luciferin goes, you will see light. A caveat of imaging in these mice is that emission from deep tissues that are harder to access is often obscured. This is particularly true in the head, where photon flux from the nose, eyes, ears, and tissue overlying the brain overshadows emission from the brain itself (Figure 2). Remarkably, it is the brain that yields the highest bioluminescence signal when these same mice are treated with luciferin amides (Figure 2).[2] This reflects both the ability of luciferin amides to traverse the blood-brain barrier, and the high selectivity for cleavage to the light-emitting luciferin by FAAH.

Ask your average medicinal chemist how to improve the cellular delivery of a carboxylic acid, and they will say: make an ester. Esters are more lipophilic, and upon entry into cells are anticipated to be hydrolyzed to the carboxylic acid by "esterase activity" (where the specific enzyme or enzymes performing this activity are often unknown). Although luciferin esters can be hydrolyzed to their respective luciferins in cells and in mice, they have not been very effective at delivering luciferins into the brain (Figure 2).[2] In part, this may be because "esterase activity" is everywhere. On the other hand, luciferin amides are exemplary at delivering synthetic luciferins into the brain, where endogenous FAAH activity liberates the parent luciferin.[2] Potentially, FAAH could be more generally exploited in a "prodrug" strategy to specifically unmask a broader range of carboxylic acids in the brain. Somewhat ironically, we find that FAAH is also responsible for some of the generic "esterase activity" toward luciferin esters, at least in tissue culture cells.[2]

Many optical imaging probes are too large or polar to efficiently enter the brain. Excitingly, bioluminescence imaging combines the specificity of a genetically encoded luciferase reporter with the versatility of a small yet highly tunable "druglike" luciferin emitter, offering a wealth of opportunities to shed light on the inner workings of the brain, and also show how to get there.

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