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Releasable Luciferin−Transporter Conjugates: Tools for the Real-Time Analysis of Cellular Uptake and Release

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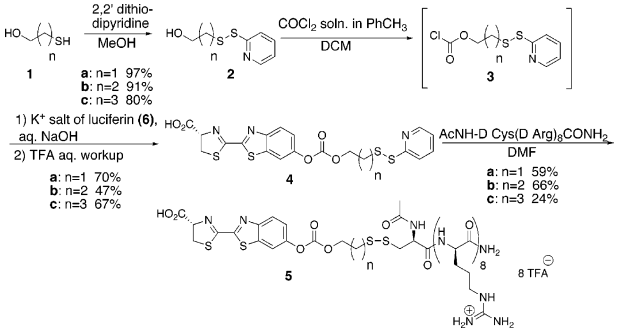
Many promising therapeutic leads fail to advance clinically due to problems with formulation and/or bioavailability. Conversion of the lead to a more polar prodrug often minimizes problems with aqueous formulation, but at the same time increases problems with passive diffusion across the relatively nonpolar membrane of cells. We previously have shown that conjugation of small molecules, peptides, proteins, nucleic acids, or imaging agents to an octaarginine or oligoguanidine transporter produces conjugates that are water soluble and readily enter cells and tissue.[1] A releasable conjugate of cyclosporin A and octaarginine has been used for intradermal delivery and advanced to Phase II clinical trials.[1a]

A major challenge confronting the further advancement of this field[2] is the development of linkers that would allow for the controllable release (if required) of a free drug/probe only after cell entry. Realization of this goal is coupled directly to the development of assays that would allow for the rapid real-time quantification of transporter−conjugate uptake and cargo release in cells and animals. Transporters covalently conjugated to fluorescent dyes can be used to measure uptake in vitro, but they cannot be used to measure cargo release in a cell or applied to real-time in vivo analyses. Radiolabeled conjugates can be used for in vivo studies, but they neither establish whether the labeled conjugate is intra- or extracellular, nor whether they are intact or have released their cargo. We have shown that intracellular cargo release is possible and measurable when an oligoarginine transporter is attached through a cysteine disulfide bond to an otherwise cell-impermeable peptide cargo.[1d] The resultant conjugate enters cells and is then cleaved to the free bioactive peptide as determined in a functional assay for ischemic damage. This assay, however, does not lend itself to rapidly evaluating new transporters, linkers, or release systems, as it is labor intensive, time consuming, difficult to quantify, and only indirectly measures release of the active cargo. To address these problems, we have developed, as reported herein, a new releasable linker system that can be used with various transporter−drug/probe conjugates and that allows for cargo release only after cell entry. A second major finding of this study is the development of an assay, based on this releasable linker and luciferin as a representative (reporter) cargo, which allows measurement of conjugate uptake and cargo release in real time in luciferase-transfected cells and potentially in transgenic animals through the emission of light, collectively emulating drug uptake, intracellular release, and receptor interaction.

A major obstacle in implementing the above strategy proved initially to be the synthetic difficulty of making luciferin conjugates. While luciferin itself has figured prominently as a research tool for decades, little is known about its modification and no information is available on its attachment to a transporter through a releasable linker. After much experimentation, a concise solution to this synthetic problem was developed, as illustrated in Scheme 1. In this route, hydroxy thiol **1** (**a**−**c**) is transformed with 2,2′-dithiodipyridine to an activated disulfide **2** (**a**−**c**). The chloroformate **3** (**a**−**c**) is then formed by reaction of disulfide **2** in CH_2Cl_2 with a solution of phosgene in toluene (20%). Due to the limited solubility of D-luciferin (**6**) in organic solvents, and to avoid protecting groups, the organic solvent is removed in vacuo and the potassium salt of luciferin is added with aqueous base to the chloroformate **3** (**a**−**c**) to form, upon acidic workup, the carbonate **4** (**a**−**c**). This carbonate serves as a reagent for conjugation to a variety of transporters. For the purposes of this inaugural study, the thiopyridyl moiety of **4** was displaced with acylated D-cysteine D-octaarginine ($AcNHcr_8CONH_2$) to give the transporter−linker conjugate **5** (**a**−**c**). The avoidance of protecting groups in this sequence provides a flexible and step economical route to these densely functionalized transporter conjugates, which bodes well for the use of this system for the synthesis and study of other transporter−linker conjugates.

Scheme 1



The stabilities of the conjugates **5a**−**c** were assayed by measuring their decomposition when incubated in Hepes buffered saline (HBS, pH 7.4) at 37 °C using analytical HPLC. The half-lives of the conjugates differed significantly, ranging from 3 h for carbonate **5a** to 11 h for carbonate **5b** to 33 h for carbonate **5c**. The decomposition products were luciferin, alcohol **7**, and CO_2 as expected from slow hydrolysis of the carbonate (Scheme 2). The pattern of increasing stability correlates with the increasing distance between the carbonyl group and the proximal sulfur atom, suggesting a role for the latter in the hydrolysis step.

To determine if release of luciferin from the conjugates could be induced by disulfide cleavage as desired for intracellular release, each conjugate was incubated with HBS pH 7.4 with the addition of 10 mM dithiothreitol (DTT). Notwithstanding their widely differing intrinsic stabilities, all three conjugates were cleaved in minutes. Free luciferin was formed without any observable intermediates in the case of carbonates **5a** and **5b**; however, with carbonate **5c**, an intermediate **8c**, whose half-life was 3 min, was

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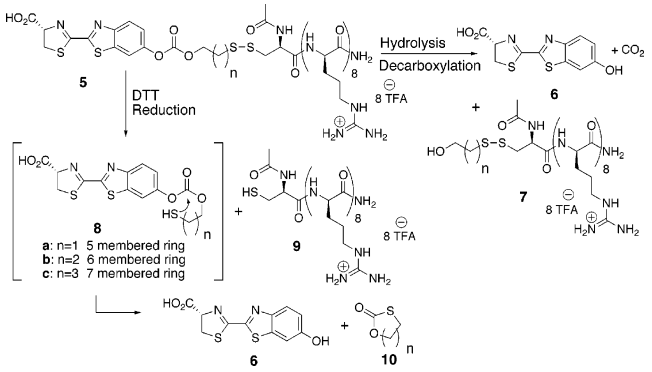
Scheme 2



observed, consistent with rapid disulfide cleavage, but slower cyclization of the intermediate thiol with the carbonate due to the required formation of a larger (seven-membered) ring.

While all three conjugates are sufficiently stable for cellular studies, only the more stable carbonates **5b** and **5c** were examined for cell uptake and release. Luciferin was used as a positive control. To study uptake and release in cell culture, varying concentrations of luciferin, **5b**, and **5c** were incubated separately with a prostate cancer cell line stably transfected with a luciferase encoding gene, PC3M-luc.[3] After an incubation time of 1 min, selected to facilitate measurement and not to maximize uptake, the cells were washed to remove any extracellular luciferin or conjugate, and the number of photons produced as a function of time was analyzed using a charge-coupled device camera (IVIS100, Xenogen Corp., Alameda, CA).[3]

The luminescent signal from cells pulsed with **5b**, which is a measure of the intracellular release of free luciferin and its turnover by intracellular luciferase, increased slightly in the first few seconds and gradually decayed, reaching background after approximately 1000 s (Figure 1). Cells treated with **5c** generated a different curve with less initial light, a slower rate of decay, and only two-thirds of the total photons produced when compared to that seen for **5b** (Figure 1). To explore whether the observed luminescence was due to intracellular release of luciferin and its reaction with luciferase and not extracellular hydrolysis of the conjugate and luciferin uptake, the experiment was repeated in Hepes buffered saline in which all sodium salts were replaced with equimolar amounts of the potassium salt (K+HBS), a condition known to eliminate the membrane potential and thereby the uptake of arginine-rich transporters but not the uptake of free luciferin.[4] Under these conditions, luminescence from the conjugate (and therefore uptake and release) was reduced by ≫90% (Figure 1), whereas luminescence from luciferin itself increased slightly (Figure 1). Consequently, the vast majority of the light arises from conjugate uptake into the cells and subsequent release of luciferin.

The different luminescence yields as a function of time observed for **5b** and **5c** is an expected consequence of the latter producing a longer-lived intermediate thiol **8c**, which not only delays release of luciferin but also could serve to reduce turnover by binding nonproductively to luciferase. There is precedent for this phenomenon in the case of the 6-O methyl ether of luciferin, which is known to bind luciferase and decarboxylate but not produce light.[5] To further explore this behavior, **5b** and **5c** (1 μM) were incubated with purified (cell-free) firefly luciferase under standard conditions with 1 mM dithiothreitol at pH 7.4, and luminescence was measured using a luminometer.[6] As with cells, **5b** produced greater luminescence than **5c**. However, an independent assay involving preincubation of each conjugate with 1 mM DTT, pH 7.4, at 37 °C for 30 min followed then by addition to luciferase resulted in

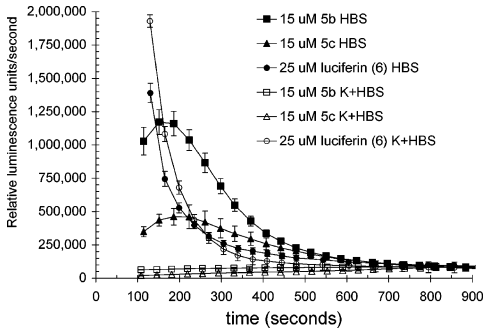


Figure 1. Real-time bioluminescence from a prostate cancer cell line stably transfected with luciferase (PC3M-luc) treated with 25 μM luciferin (**6**) or 15 μM releasable luciferin conjugates, **5b** or **5c**, in either HBS or K+HBS. For the total luminescence see Supporting Information.

equivalent luminescence from both conjugates. Thus, the lower signal arising from **5c** is consistent with the generation and interference of a longer-lived intermediate under the short time course of the experiments. Both conjugates ultimately release the luciferin cargo efficiently.

The synthesis and performance of these releasable luciferin conjugates establishes an operationally facile method to quantify in real time uptake and release of new or established transporters and linkers in a cellular assay that emulates drug−conjugate delivery into a cell, drug release, and drug turnover by an intracellular target. The new disulfide−carbonate linker system can be tuned for stability (from hours to days) without affecting its rapid rate of cargo release (minutes) in cells through disulfide cleavage. The linker−luciferin system was designed to allow quantification of uptake of various transporters in both transfected cells and transgenic animals, thereby providing a facile method to measure and correlate in vitro and in vivo activities in real time. Studies on uptake in transgenic animals are in progress.

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Supporting Information Available: Experimental results and procedures for the synthesis and assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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