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Cell uptake and tissue distribution of radioiodine labelled D-luciferin: implications for luciferase based gene imaging

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Summary

Optical luciferase gene imaging is emerging as a method to monitor gene expression in small animals. However, there is concern over how regional availability of exogenously administered substrate may affect photon emission. We thus synthesized [^{125}I]iodo-D-luciferin, which demonstrated substrate characteristics for firefly luciferase, and investigated its cell uptake kinetics and *in vivo* biodistribution. Luminescence assays of *luc* gene transduced cells confirmed a linear decline in emitted light units with decreasing luciferin concentration. Both *luc* gene transduced and control cells demonstrated a low level of cellular uptake and rapid washout of [^{125}I]iodo-D-luciferin, although early uptake was slightly higher for transduced cells ($P < 0.005$). Biodistribution in ICR mice demonstrated that early uptakes in liver, lung, myocardium and muscle were lower with intraperitoneal compared to intravenous administration. In view of the poor cell uptake, uptake levels (<3%ID/g) suggest that substrate concentration may limit light emission rates in organs such as bone, muscle, myocardium, and particularly the brain. Thus, substrate availability should be considered as a potential limiting factor for photon emission efficiency in certain organs when attempting quantitative interpretation of optical *luc* gene imaging. (© 2003 Lippincott Williams & Wilkins)

Keywords: luciferase, D-luciferin, gene imaging.

Introduction

Non-invasive monitoring of gene expression in living subjects after gene transfer is an active area of current molecular imaging research [1, 2]. Radiotracer methods for reporter gene imaging using positron emission tomography and single photon emission computed tomography have been extensively investigated and validated [3–5]. More recently, improvements in optical imaging techniques have led to the emergence of luciferase based reporter systems as a promising alternative for real time gene imaging in small animals [6, 7]. The firefly luciferase, encoded by the *luc* gene, catalyses the oxidation of D-luciferin (D-(-)-2-(6′-hydroxy-2′-benzothioazolyl)thiazoline-4-carboxylic acid) in the presence of ATP to generate visible light signals. The *luc* gene is a popular reporter gene widely utilized in molecular biology experiments for *in vitro* transgene expression assays. These assays exploit the fact that light output is proportional to the concentration of luciferase enzyme when substrate is provided in excess [8]. Recently, it has been revealed that the *luc* system can also be applied to living animals by utilizing charged coupled device (CCD) cameras, which can detect photons emitted from tissue with high sensitivity. The system has been successful in imaging gene expression in various organs transduced with the *luc* gene following systemic administration of D-luciferin [9–12].

However, since reaction rates regulated by enzyme kinetics are proportional to local substrate concentration, assessment of gene expression using optical *luc* gene imaging is dependent on the assumption that exogenously administered substrate is delivered to organs of

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interest is in sufficient and comparable levels. But as the actual kinetics and biodistribution of D-luciferin have not been elucidated to date, a concern for *luc* gene imaging is whether the photon flux faithfully reflects the regional luciferase expression level, or if local substrate delivery and permeability significantly affects luminescence intensity [13]. We thus investigated the uptake characteristics of radioiodine labelled D-luciferin in *luc* gene transduced cells and patterns of its *in vivo* biodistribution in rodents after intraperitoneal or intravenous administration.

Material and methods

In vitro *gene transfer and luminescent luciferase assays*

COS-7 cells, obtained from the American Type Culture Collection (Rockville, MD) were cultured in RPMI 1670 media (Gibco BRL) supplemented with 10% fetal bovine serum and antibiotics (Gibco BRL). Gene transfer was achieved by calcium phosphate precipitation in 80% confluent COS-7 cells using pGL_3–Luc plasmid DNA (Promega, Madison, WI), which contains the luciferase gene driven by a cytomegalovirus promoter. Control cells were transduced with the empty pGL_3 control plasmid (Promega, Madison, WI).

At 48 h after gene transfer, cells were harvested with trypsination, washed with phosphate buffered saline (PBS), and transferred to culture tubes for assays and uptake experiments. Light emission was quantified as the summation of luminometer detected light units during the second minute of reaction between intact cells or cell lysates with D-luciferin in reaction buffer from a commercial assay kit (Stratagene, La Jolla, CA). To investigate the effect of D-luciferin concentration on emitted light intensity, measurements were repeated with up to 10^4-fold serial dilutions from an initial concentration of 1 μM. Separate assays were performed with cells transduced with DNA loads ranging from 0.1 to 10 μg per 100 mm plates.

Radioiodine labelling of D*-luciferin and cell uptake experiments*

[$^{123/125}$I]Iodo-D-luciferin was prepared by electrophilic radioiodination of D-luciferin with Na[$^{123/125}$I]I in the presence of chloramine-T. The product was purified by HPLC with a radiochemical yield of 35–50%, and radiochemical purity of 99%. The final product was appropriately diluted with saline and used for experiments. We also synthesized cold iodine labelled D-luciferin (iodo-D-luciferin) by by the same method as for [$^{123/125}$I]iodo-D-luciferin, except that NaI was used in place of radioiodide. This compound was used to examine whether iodine labelled D-luciferin retained substrate specificity for firefly luciferase, based on luminescent luciferase assays.

For cell uptake experiments, control COS-7 cells and cells expressing the *luc* gene were incubated with 148 kBq (4 μCi) [^{125}I]iodo-D-luciferin at 37°C in 500 μl PBS (pH 7.4). After incubation for 10–120 min, cell pellets were rapidly washed twice with ice cold PBS and measured for radioactivity on a well type gamma counter.

Biodistribution studies and animal imaging

Unanaesthetized normal male ICR mice of approximately 30 g were intraperitoneally injected with 740 kBq (20 μCi) of [^{125}I]iodo-D-luciferin alone or mixed with 126 mg·kg^{-1} body weight doses of unlabelled D-luciferin, and killed by cervical dislocation 5 min later (each $n = 2$). Separate groups of mice were injected with radiotracer (mixed with 126 mg·kg^{-1} D-luciferin) either intraperitoneally or intravenously via the tail vein, and were killed in triplicates at 5 and 45 min after injection. Blood was sampled and major organs were promptly excised, weighed and measured for radioactivity on a gamma counter. Uptakes for each organ were expressed as per cent injected dose per gram of tissue.

For imaging, normal male Sprague–Dawley rats, anaesthetized by using xylazine/ketamine, were injected with 7.4 MBq (200 μCi) of [^{123}I]iodo-D-luciferin either intravenously or intraperitoneally. Serial 5 min scintigraphic images were acquired starting from 5 min to 1 h post-injection. A gamma camera (Triad XLT, Trionix Research Laboratory, Ohio) with a parallel hole collimator and a 15% energy window centred around 160 keV was used, and data were stored on a 128×64 pixel sized matrix. All animal handling and procedures were consistent with the guide for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee.

Results

COS-7 cells transduced by the *luc* gene expressed high luciferase activity at 48 h, as measured by luminescent luciferase assays of intact cells and cell lysates. However, when assays on intact cells were performed with serially diluted substrate, the light emission measured showed a linear decrease as D-luciferin concentration was reduced from 1 μM to 0.1 nM (Fig.1(A)). The substrate concentration effect on photon emission was observed for cells transduced with either 10 μg or 1 μg of pGL_3–Luc plasmid (Fig. 1(B)). These results show that, under these conditions, the amount of photons emitted from intact

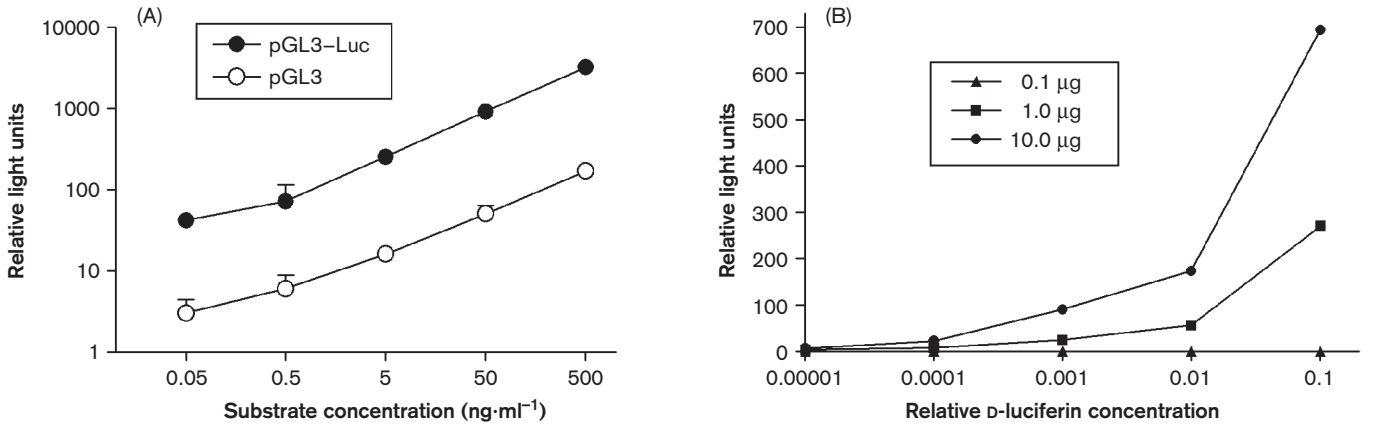

Fig. 1. (A) Relation between D-luciferin concentration and relative light units detected. pGL3–Luc contains a luciferase gene driven by the cytomegalus virus promoter, while the control pGL3 does not contain a gene insert. Data are presented as mean ± SD of triplicates. (B) Relation between D-luciferin concentration and relative light units from cells transduced with different DNA loads of pGL3–Luc.

cells that express luciferase is significantly affected by the local availability of D-luciferin.

We then investigated the cellular handling and *in vivo* biodistribution of D-luciferin by utilizing [^{125}I]iodo-D-luciferin as its radiolabelled tracer (Fig. 2). Kinetics studies using iodo-D-luciferin demonstrated that the compound retained characteristics as a substrate for luciferase enzyme. During luminescent luciferase assays, the addition of iodo-D-luciferin to purified luciferase enzyme and ATP led to light emissions whose intensity correlated to enzyme concentration (data not shown). When the cellular handling of [^{125}I]iodo-D-luciferin was investigated there was poor uptake of radiotracer in untransduced control cells at 30 min ($0.13 \pm 0.00\%$ of applied activity). Cells transduced by the *luc* gene showed a statistically significant, but small, increase of uptake compared to control cells ($0.18 \pm 0.00\%$, $P < 0.005$; Fig. 3(A)). However, radioactivity rapidly decreased from 30 to 60 min of incubation for both control cells and cells that expressed the *luc* gene (Fig. 3(B)), indicating that both D-luciferin and its hydrolysed product are poorly retained within cells.

Comparison of the 5 min biodistribution in mice that had received [^{125}I]iodo-D-luciferin i.p., with or without 126 mg·kg^{-1} of D-luciferin, demonstrated a difference in distribution pattern, which showed significantly higher blood, myocardium and hepatic activity in the presence of carrier D-luciferin (Fig. 4). Hence, the remaining experiments were performed with radiotracer mixed with carrier D-luciferin. After i.p. injection, blood activity was relatively high at 5 min (15.7 ± 0.7%ID/g), but cleared to 6.7 ± 2.4%ID/g by 45 min. There was high uptake in the liver and kidneys, suggesting both hepatic

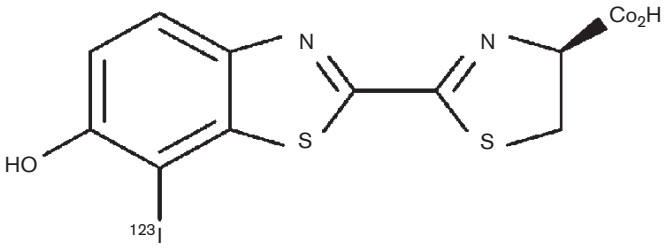

Fig. 2. Chemical structure of ^{123}I labelled iodo-D-luciferin.

and renal excretion as the route for radiotracer elimination. The intestines, stomach and pancreas had high uptakes, but presumably due to direct absorption or adsorption of the radiotracer from the peritoneum. The lungs, myocardium, bone and muscles had lower uptake levels (1.8–4.5 and 1.6–2.8%ID/g at 5 and 45 min, respectively). Particularly notable was the remarkably low level of uptake in the brain (Table 1).

Intravenous injection of [^{125}I]iodo-D-luciferin resulted in a biodistribution significantly different in pattern compared to that after intraperitoneal administration. Blood activity was 2-fold higher at 5 min after intravenous injection, but cleared more rapidly. Early uptake was higher in the myocardium, lung, liver and muscle, while it was lower in the gastrointestinal organs, pancreas and spleen compared to that with intraperitoneal injection. Brain uptake remained very low regardless of the administration route or time points (Table 1). Serial images in rats of that received [^{123}I]iodo-D-luciferin by i.v. and i.p. injection demonstrated findings consistent with mouse biodistribution results (Fig. 5).

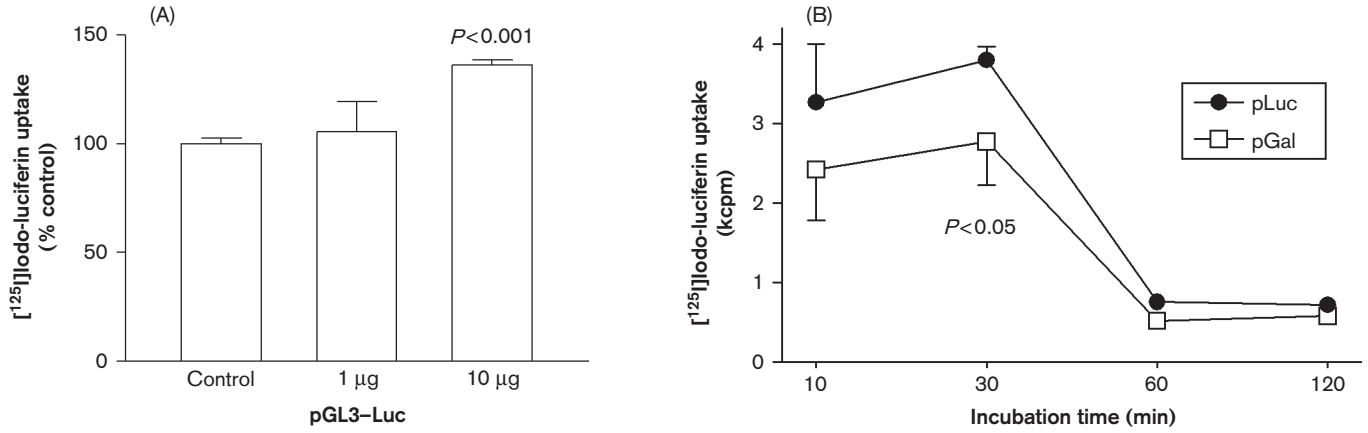

Fig. 3. (A) DNA dose-dependent uptake of [^{125}I]iodo-D-luciferin in pGL3–Luc plasmid transduced cells at 30 min of incubation. (B) Time-dependent uptake of [^{125}I]iodo-D-luciferin in cells transduced by pGL3–Luc or control plasmid. All data are presented as mean ± SD of triplicates.

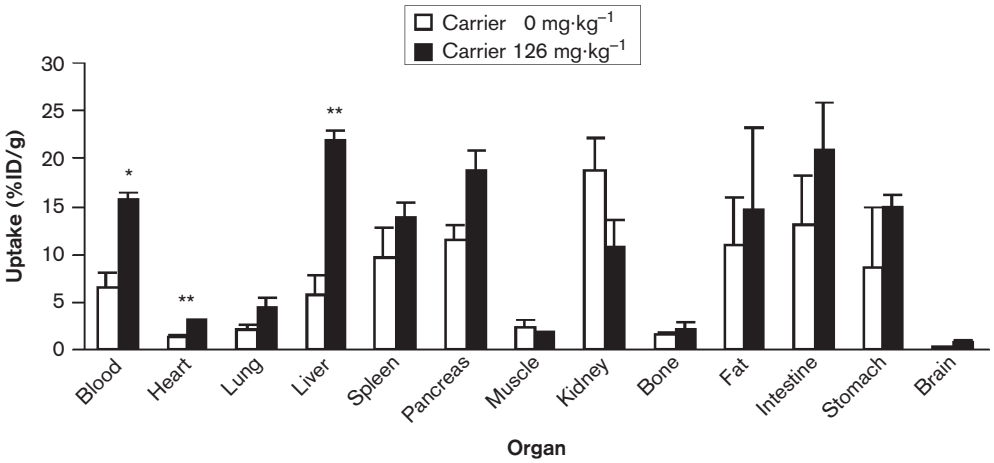

Fig. 4. Biodistribution of [^{125}I]iodo-D-luciferin 5 min after intraperitoneal injection in normal ICR mice. Light and dark bars denote radiotracer mixed with 0 and 126 mg·kg^{-1} of unlabelled D-luciferin, respectively. *$P < 0.05$; **$P < 0.01$.

Table 1. Biodistribution of [^{125}I]iodo-D-luciferin in mice.

	Intravenous		*Intraperitoneal*		P *value*	
Organ	*5 min*	*45 min*	*5 min*	*45 min*	*5 min*	*45 min*
Blood	32.2 ± 7.6	5.8 ± 1.7	15.7 ± 0.7	6.7 ± 2.4	NS	NS
Myocardium	9.4 ± 1.2	1.2 ± 0.5	3.0 ± 0.1	2.3 ± 0.1	< 0.02	< 0.05
Lung	13.2 ± 1.1	2.7 ± 1.1	4.5 ± 0.9	3.8 ± 0.5	< 0.02	NS
Liver	31.9 ± 0.2	3.7 ± 1.2	21.9 ± 0.9	9.7 ± 0.9	< 0.005	< 0.02
Spleen	5.7 ± 0.0	1.6 ± 0.8	13.9 ± 1.6	5.3 ± 0.6	< 0.02	< 0.02
Pancreas	6.9 ± 3.5	2.8 ± 0.8	18.7 ± 2.1	9.3 ± 0.4	< 0.02	< 0.005
Muscle	3.5 ± 0.1	1.0 ± 0.5	1.8 ± 0.1	1.6 ± 0.3	< 0.005	NS
Kidney	15.1 ± 3.1	8.8 ± 0.4	10.6 ± 3.0	17.5 ± 1.6	NS	< 0.005
Bone	3.9 ± 0.4	1.6 ± 0.7	2.2 ± 0.8	2.0 ± 0.6	NS	NS
Intestine	10.1 ± 0.4	3.5 ± 1.0	20.8 ± 4.9	38.7 ± 11.7	NS	< 0.01
Stomach	6.7 ± 0.0	7.6 ± 2.7	14.8 ± 1.3	14.0 ± 4.4	< 0.02	NS
Brain	0.4 ± 0.1	0.5 ± 0.4	0.7 ± 0.2	0.4 ± 0.1	NS	NS

NS, not significant. Data are presented as %ID/g in mean ± SD of triplicate animals.

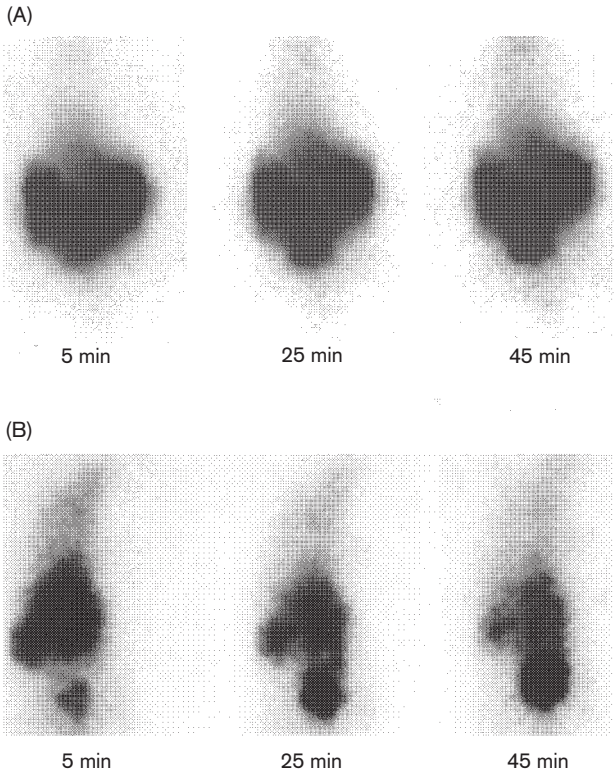

Fig. 5. Serial scintigraphic images of normal Sprague–Dawley rats after (A) intraperitoneal or (B) intravenous injection of [^{123}I]iodo-D-luciferin.

Discussion

Optical *luc* reporter gene imaging of gene expression in living rodents requires the assumption that light intensity faithfully reflects the level of regional transgene expression. Such an assumption is valid for *in vitro* luciferase assays, where detected light units provide accurate quantitative measurements of *luc* gene expression. A linear relationship between enzyme concentration and reaction rate is made possible through controlled reaction conditions, which include provision of D-luciferin substrate in excess levels [14]. D-luciferin is generally used in concentrations of about 200 μM, and luminescence intensities are proportional to luciferase concentrations in the picogram to nanogram range.

Meanwhile, for a fixed concentration of enzyme, a decrease in local substrate concentration will cause a decline in the reaction rate in accordance with Michaelis–Menten kinetics. The reaction rate would reach half its maximum value when D-luciferin concentrations approximate its K_m (Michaelis constant) value (8 μM), and would further decrease linearly with more reduction of substrate concentration [15]. Our results confirm a linear relationship between measured relative light units and substrate concentrations of approximately 0.1 nM to 1 μM (0.5 ng·ml^{-1} to 0.5 μg·ml^{-1}). A similar finding has been shown for light response from *Renilla* luciferase activity with colenterazine concentrations ranging from 0.1 to 10 μg·ml^{-1} [13].

This implies that for optical *luc* imaging, tissues with identical levels of luciferase expression could show different luminescence, depending on local availability or biodistribution of administered D-luciferin. Despite such concern, previous studies have repeatedly demonstrated successful imaging of photon signals from a variety of organs after *luc* gene transfer. Indeed, CCD cameras have allowed imaging of *luc* gene expression in the liver, lungs, bone, bone marrow, teeth, salivary gland, prostate, testis, bladder, subcutaneous tissue [9], skeletal muscle [11] and myocardium [12]. Thus, systemically administered D-luciferin does reach organs of interest in sufficient concentrations to generate detectable light emission. This is not unexpected, since we observed detectable photon emission when D-luciferin at concentrations as low as 50 pg·ml^{-1} was mixed with *luc* transduced cells. However, the question still remains concerning whether the magnitude of detected light is a dependable index for quantitative assessments of regional *luc* gene expression.

D-luciferin is notorious for its poor permeability through cell membranes [8], which has led to the need for special methods when measuring *luc* activity within intact cells, such as a low pH buffer to cause protonation of the carboxyl group of D-luciferin [16], or the use of dimethyl sulfoxide (DMSO) to permeabilize the cell membrane. The very low cellular uptake of radioiodine labelled D-luciferin in our study supports the poor cell membrane permeability of D-luciferin. It can be pointed out that iodo-D-luciferin may not be an exact tracer for D-luciferin. However, this is a potential limitation for most radiolabelled tracers. Moreover, we were able to show that iodo-D-luciferin partially retained substrate characteristics of D-luciferin for luciferase enzyme, which confirms that the chemical characteristics are partially retained.

Our biodistribution results of i.p. administrated radioiodine labelled D-luciferin show 5 min uptake levels in major organs such as the myocardium, skeletal muscle, and bone within the 1–3%ID/g range. For a 30 g mouse injected with a dose of 100 mg·kg^{-1}, this roughly translates into tissue concentrations of 60–180 μM, which at a glance appear sufficient. However, there is an important difference between biodistribution and actual intracellular availability of a given substrate, which is largely because only a portion of the substrate distributed to an organ of interest can actually permeate through the cell membrane and interact with their intracellular enzyme counterparts. Judging from the very poor uptake

rates shown on cell experiments, the actual intracellular substrate concentration is likely to be much lower than that inferred from radiotracer distribution results alone. Moreover, since our biodistribution results show blood activity to be several-fold higher than the activity of various tissues, blood activity within the tissue sample should account for a substantial portion of the measured tissue radioactivity. Taken together, these facts and the actual biodistribution data indicate that intracellular D-luciferin concentration in various tissues is likely to be within the range for reaction rate to be significantly influenced by substrate concentration.

The reason for higher tissue uptake of carrier added compared to carrier free radiolabelled luciferin is not clear and can only be speculated. While renal excretion appears to be the major route for radiolabelled luciferin elimination (as shown on serial imaging), the kidneys were the sole organ whose radioactivity was higher when carrier free radiolabelled luciferin was used. Hence, one plausible explanation is that carrier doses of unlabelled luciferin decreased renal excretion of radiolabelled luciferin, thereby allowing more radiotracers to distribute to other tissues.

There was also substantial difference in biodistribution between intraperitoneal and intravenously injected radioiodine labelled D-luciferin, which may have practical bearing depending on the organ of interest. A particularly prominent finding in the biodistribution study was the very low activity in the brain after either intraperitoneal or intravenous administration of iodine labelled D-luciferin, which was up to 20-fold lower than that of the liver at 5 min. This suggests that optical *luc* gene imaging may substantially underestimate transgene expression levels in the brain. In a study on non-invasive imaging of tetracycline inducible *luc* gene expression in mouse liver and brain, Hasan *et al*. estimated that imaging efficiency in the brain is approximately 200-fold lower than that in the liver [17]. The authors attributed the findings to the possibility that the skull serves as a barrier for external transmission of generated photons. However, it has been shown that the bone is not a major barrier for optical luciferase imaging [18]. Our distribution results indicate that limited substrate delivery may have significantly contributed to the observed poor imaging efficiency for gene transfer to the brain.

Our results suggest that tissue difference in D-luciferin delivery may influence the intensity of photon emission depending on the organs of interest. Thus, investigators attempting to compare luciferase gene expression levels with optical imaging may need to control for potential confounding effects caused by regional difference in D-luciferin availability. Photon intensities from the same tissue in a basal state, for instance, could serve as a self-control for measuring temporal or post-manipulation changes of expression levels within a particular tissue of interest. When gene expression levels between different organs need comparison, one may need to first obtain basic expression level data from luminescent assays of homogenized tissue samples, which could then be used to correct for observations made from *in vivo* studies. In any event, substrate biodistribution should be considered when attempting quantitative measurements of *luc* gene expression between different tissues using optical imaging.

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