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PET imaging and optical imaging with D-luciferin [^{11}C]methyl ester and D-luciferin [^{11}C]methyl ether of luciferase gene expression in tumor xenografts of living mice

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Abstract—New carbon-11 labeled D-luciferin analogs D-luciferin [^{11}C]methyl ester ([^{11}C]LMEster, [^{11}C]**1**) and D-luciferin [^{11}C]methyl ether ([^{11}C]LMEther, [^{11}C]**2**) were synthesized in 25–55% radiochemical yield. PET studies with [^{11}C]LMEster and [^{11}C]LMEther demonstrate a lower retention of the C-11 label at 45 min post-injection in luciferase expression tumor. Optical imaging with unlabeled substrate D-luciferin and radiotracers [^{11}C]LMEster and [^{11}C]LMEther gave tumor luciferase images within a few minutes of photon counting.

Cancer is commonly treated by various combinations of surgery, radiation therapy, chemotherapy, hormone therapy, shockwave therapy, and gene therapy. Gene therapy has received wide attention as a potential method in treating several common cancers such as breast cancer and prostate cancer.[1,2] A key step in gene therapy and transgenic animal study is the ability to monitor the extent of transgene expression. The classic methods of assaying transgene expression require biopsies or death of the subject. Therefore, it is very important to develop techniques to non-invasively and repetitively determine the location, duration, and magnitude of transgene expression in living animals.[3] Non-invasive monitoring of gene expression in living subjects after gene transfer is an active area of current molecular imaging research.[4] Radiotracer methods for reporter gene imaging using biomedical imaging modalities such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) have been extensively investigated and validated, since both PET and SPECT have sufficient sensitivity and quantitation to measure the expression of genes in vivo.[5] Our purpose is to develop gene reporter probes for in vivo PET imaging technique to non-invasively, quantitatively, and repetitively image reporter gene expression in cancer murine viral gene transfer and transgenic models. In our previous work, we have developed and synthesized a series of PET reporter probes that target herpes simplex virus type 1 thymidine kinase (HSV-tk) gene.[6–10] In this ongoing study, we targeted another popular reporter gene luciferase and developed carbon-11 radiolabeled substrates D-luciferin [^{11}C]methyl ester (D-(−)-2-(6′-hydroxy-2′-benzothioazolyl)thiazoline-4-carboxylic acid [^{11}C]methyl ester, [^{11}C]LMEster, [^{11}C]**1**) and D-luciferin [^{11}C]-methyl ether (D-(−)-2-(6′-[^{11}C]methoxy-2′-benzothioazolyl)thiazoline-4-carboxylic acid, [^{11}C]LMEther, [^{11}C]**2**) as new potential gene reporter probes for luciferase. We also studied luciferase based gene imaging modalities with optical imaging administered unlabeled substrate D-luciferin (D-(−)-2-(6′-hydroxy-2′-benzothioazolyl)thiazoline-4-carboxylic acid) and radiotracers [^{11}C]LMEster and [^{11}C]LMEther, and PET imaging coupled with reporter probes [^{11}C]LMEster and [^{11}C]LMEther in tumor xenografts of living mice.

Luciferase enzyme is highly selective to convert substrate D-luciferin in the presence of oxygen and ATP (adenosine triphosphatase) to produce bioluminescence, which has been used for real-time, low-light imaging of

Keywords: D-Luciferin [^{11}C]methyl ester ([^{11}C]LMEster, [^{11}C]**1**); D-Luciferin [^{11}C]methyl ether ([^{11}C]LMEther, [^{11}C]**2**); Luciferase; Gene expression; Positron emission tomography (PET) imaging; Optical imaging; Tumor xenografts.
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gene expression in cell cultures, individual cells, whole organisms, and transgenic organisms.[11–14] Bioluminescence in the firefly is a complex phenomenon based on a simple set of chemical reactions of D-luciferin, ATP, oxygen, and magnesium ion catalyzed by the enzyme luciferase.[15] Two steps are involved: (1) the substrate D-luciferin reacted with ATP and magnesium ion catalyzed by the enzyme luciferase to form an enzyme–substrate–AMP (adenosine monophosphate) complex and inorganic pyrophosphate (PPi); (2) this complex reacted with oxygen to form oxyluciferin and AMP, and to emit light (hν).

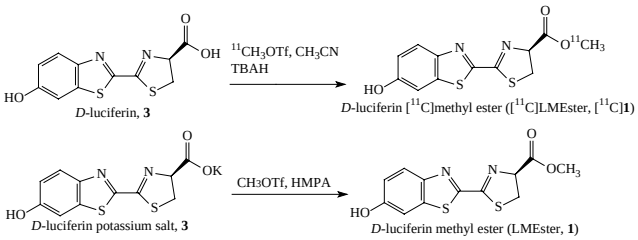
The luciferin/luciferase bioluminescence imaging works, but it is not certain to what degree the high contrast images obtained are the result simply of selective substrate conversion or might be dependent upon selective substrate distribution. The latter issue, selective distribution, is one that can be approached by PET imaging. Then, we prepare luciferin substrate analogs that can be labeled with C-11 so that their distribution could be followed by PET. Assuming, or better yet, establishing that these luciferin analogs have similar capacity to interact with luciferase, then their distribution, determined by PET, would provide evidence for the degree to which the bioluminescence images resulted from selective substrate consumption or selective substrate distribution. The outcome of PET imaging, if there is no selective substrate distribution to the luciferase-containing tumors, would establish that the bioluminescence images are solely or largely the result of selective substrate consumption.

To develop radiotracer methods for imaging reporter gene expression, we designed and synthesized a series of radiolabeled D-luciferin analogs. The structure modifications of the D-luciferin for SPECT and PET tracers development could be at the hydroxyl group, benzothioazolyl group, and carboxylic acid group. Radioiodine labeled D-luciferin analogs 7′-[$^{123/125}$I]iodo-D-luciferin, in which the iodine-123/125 labeled structure modifications are at the benzothioazolyl group, have been reported as reporter probes for SPECT imaging of luciferase gene expression; however, the cell uptake and tissue biodistribution results of 7′-[$^{123/125}$I]iodo-D-luciferin were disappointing, which showed low level of cellular uptake and rapid washout of the tracer.[5,16] Therefore, we consider if a positron labeled agent with PET works for luciferase imaging, and we investigated the development of carbon-11 labeled D-luciferin analogs for PET imaging modality. Consequently, we designed carbon-11 labeled structure modification at the carboxylic acid group to make [^{11}C]LMEster, [^{11}C]**1**, and carbon-11 labeled structure modification at hydroxyl group to make [^{11}C]LMEther, [^{11}C]**2**.

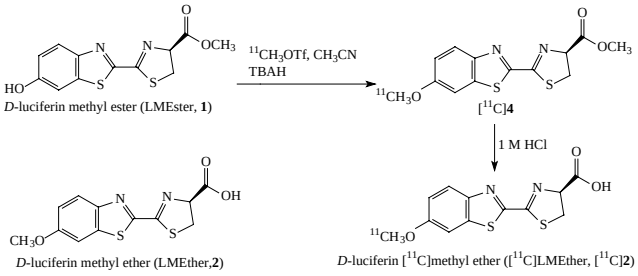
The unlabeled D-luciferin analogs D-luciferin methyl ester (LMEster, **1**)[15] and D-luciferin methyl ether (LMEther, **2**)[17] have been reported to have similar in vitro binding properties with those of D-luciferin in the bioluminescence and to undergo the same reaction mechanism to produce the bioluminescence. This provides the rationale for the synthesis of radiolabeled D-luciferin analogs [^{11}C]LMEster and [^{11}C]LMEther. Both D-luciferin and LMEther are commercially available. D-luciferin is an important starting material for the structure modifications, and LMEther is one of the reference standards for [^{11}C]LMEther. Thus, we turn our chemistry effort toward the synthesis of LMEster, which serves as another reference standard for [^{11}C]LMEster, also as a precursor for the tracer [^{11}C]LMEther.

The synthesis of LMEster, **1** and [^{11}C]LMEster, [^{11}C]**1** is shown in Scheme 1. Commercially available starting material D-luciferin (**3**) was methylated with [^{11}C]CH_3OTf[18] under basic conditions using TBAH through the *O*-[^{11}C]methylation method. The pure target tracer [^{11}C]LMEster was isolated by solid-phase extraction (SPE) purification[19] using a C-18 Sep-Pak cartridge in 40–55% radiochemical yield based on $^{11}CO_2$, decay corrected to end of bombardment (EOB), and 15–20 min synthesis time. The methylation of D-luciferin was employed in the synthesis of unlabeled reference standard LMEster. Commercially available D-luciferin potassium salt (**3**) was reacted with CH_3OTf catalyzed by HMPA to give LMEster in 63% chemical yield. Chemical purity, radiochemical purity, and specific radioactivity were determined by analytical HPLC methods.[20] Retention times were: t_R**3** = 2.03 min and t_R[^{11}C]**1** = 4.38 min. The chemical purity of reference standard **1** was >97%. The radiochemical purity of target tracer [^{11}C]**1** was >95%, and the specific activity of the tracer [^{11}C]**1** was >1.0 Ci/μmol at end-of-synthesis (EOS).

The synthesis of [^{11}C]LMEther, [^{11}C]**2** is shown in Scheme 2. The precursor LMEster, **1**, was methylated by [^{11}C]CH_3OTf to provide a single [^{11}C]methylated LMEster intermediate ([^{11}C]**4**), which was isolated by the C-18 SPE procedure from the radiolabeling mixture and quickly followed a deprotection reaction[21] with 1 M



Scheme 1. Synthesis of [^{11}C]LMEster.



Scheme 2. Synthesis of [^{11}C]LMEther.

HCl to give the target tracer [^{11}C]LMEther in 25–35% radiochemical yield based on $^{11}CO_2$, decay corrected to EOB. The large polarity difference between the phenol precursor and the labeled ether product permitted the use of an efficient SPE technique for purification of radiolabeled intermediate [^{11}C]**4**. The reaction mixture was diluted with $NaHCO_3$ (0.1 M) and loaded onto C-18 cartridge by gas pressure. The cartridge column was washed with water to remove unreacted phenol precursor, unreacted [^{11}C]CH_3OTf, and reaction solvent acetonitrile, and then intermediate [^{11}C]**4** was eluted with ethanol. The evaporation under vacuum gave [^{11}C]**4** crude product, which was hydrolyzed by 1 M HCl and concentrated under vacuum to give pure target compound [^{11}C]**2**. Chemical purity, radiochemical purity, and specific radioactivity were determined by analytical HPLC methods as aforementioned. Retention times were: t_R**1** = 4.38 min, t_R[^{11}C]**4** = 6.71 min, and t_R[^{11}C]**2** = 2.68 min. The chemical purity of reference standard **2** was >97%. The radiochemical purity of target tracer [^{11}C]**2** was >93%, and the specific activity of [^{11}C]**2** was >1.0 Ci/μmol at EOS.

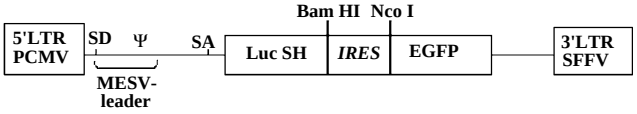
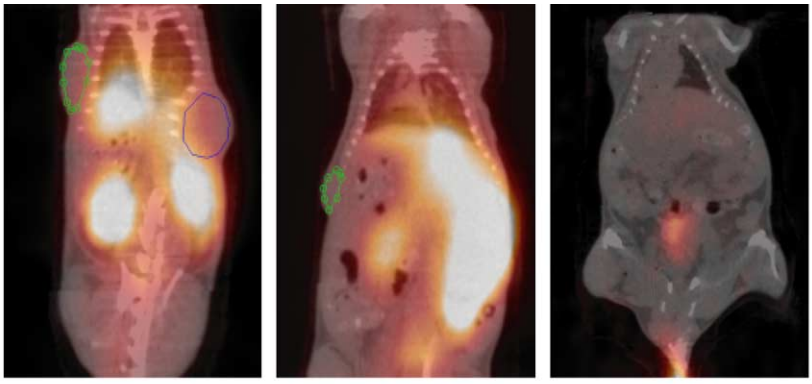


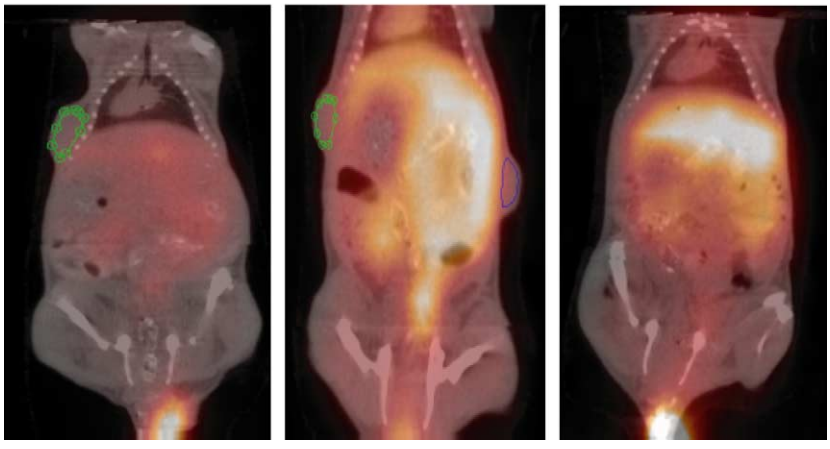
Figure 1. Schematic representation of the SF91–LucSH–EGFP retrovirus vector.

The tumor xenografts of living mice were NOD/SCID mice implanted with SKOV-3x tumor cells, and SKOV-3x tumor cells transduced with a retroviral vector, SF91–LucSH–EGFP, that co-expresses the firefly luciferase and the enhanced green fluorescent protein (EGFP) as shown in Figure 1.[22] The SKOV-3x tumor without luciferase expression was located at the right-hand side of the mouse body and served as control tumor. The SKOV-3x tumor transduced with SF91–LucSH–EGFP was located at the left-hand side of the mouse body and served as luciferase-expressing tumor.

In vivo dynamic PET imaging studies[23] of tracers [^{11}C]LMEster and [^{11}C]LMEther in tumor mice and control mouse without tumor were performed in Indy-PET-II scanner[24] for 60 min post-intravenous injection of 0.2 mCi of the tracer in a mouse, and the images are shown in Figure 2. All PET images are coronal view, and overlaid with μ-CT. The location of the luciferase



[^{11}C]LMEster PET/μ-CT images in tumor mice 1 and 2, and control mouse (from left to right)



[^{11}C]LMEther PET/μ-CT images in tumor mice 1 and 2, and control mouse (from left to right)

Figure 2. PET/μ-CT overlay images of tracers [^{11}C]LMEster and [^{11}C]LMEther in NOD–SCID tumor-bearing mice and control mouse anesthetized with acepromazine (0.2 mg/kg, im) and torbugesic (0.2 mg/kg, im), administered with 0.2 mCi radioactivity, and scanned with IndyPET-II for 60 min. The images are coronal views in which the location of the luciferase expression SKOV-3x tumor is indicated in the green circle region, and the location of the control SKOV-3x tumor is indicated in the blue circle region.

expression SKOV-3x tumor is indicated in the green circle region, and the location of the control SKOV-3x tumor is indicated in the blue circle region. We have developed a method[25] for registration and fusion of small animal scans acquired with the IndyPET-II scanner and the EVS RS-9 CT scanner, which can provide vital information about the anatomic information of tissues and organs in small animals. The μ-CT uses X-rays that pass through the animal to obtain structural information of the animal. It is an ideal instrument for biomedical research laboratories to non-destructively acquire 3-D images of both in vivo and in vitro specimens. The X-ray radiation that is imposed on the animal is minimum and does not result in any physiology change. The registration and fusion of EVS μ-CT with IndyPET-II will help to draw the regions of interest (ROIs) of the images. All images were acquired in list-mode and sorted into 15×20 s frames, 10×60 s frames, and 9×300 s frames. Images were reconstructed using filtered back projection with a 70% Hanning filter (4.242 cm^{-1} cutoff frequency). The PET/μ-CT images of the tracers [^{11}C]LMEster and [^{11}C]LMEther from tumor mice 1 and 2 in Figure 2 showed both control tumor and luciferase tumor were invisible with the tracer. PET imaging with the tracers [^{11}C]LMEster and [^{11}C]LMEther was unable to locate the tumor luciferase uptake and showed poor cell uptake, which are consistent with the results from 7′-[$^{123/125}$I]iodo-D-luciferin previously reported by Lee et al.[5,16]

The dynamic PET data of tracers [^{11}C]LMEster and [^{11}C]LMEther are shown in Figures 3 and 4. The plot Figure 3 is the average left tumor (luciferase), right tumor (control), and muscle intensity (*y*-axis) versus time (*x*-axis) from injection, which indicates the kinetics of the tracer [^{11}C]LMEster in 2 tumor mice at each time point in 60 min of entire scan time. Similarly, the plot Figure 4 is the average left tumor (luciferase), right tumor (control), and muscle intensity (*y*-axis) versus time (*x*-axis) from injection, which indicate the kinetics of the tracer [^{11}C]LMEther in 2 tumor mice at each time point in 60 min of entire scan time. The SKOV-3x (right): SKOV-3x [SF91–LucSH–EGFP] (left) tracer retention ratio at 45 min post-injection was 1.14 ± 0.11 and 2.08 ± 0.55 for [^{11}C]LMEster and [^{11}C]LMEther, respectively. The PET data show there are less uptakes of C-11 tracers in the gene-expressing tumor than in the non-expressing tumor and demonstrate there is no selective radiolabeled substrate distribution to the luciferase-containing tumors.

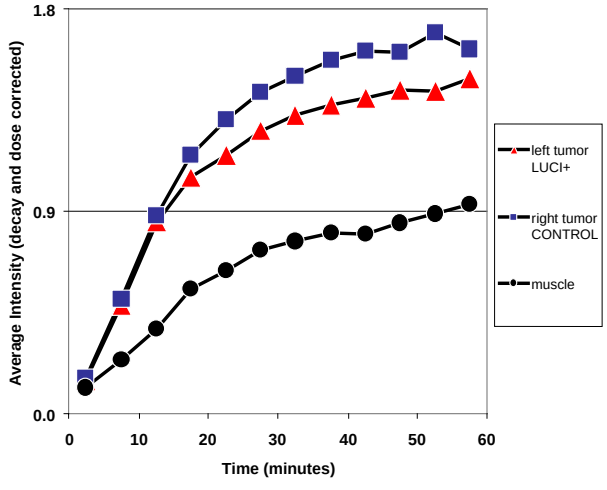


Figure 3. Average left tumor (luciferase), right tumor (control) and muscle intensity versus time from injection of the tracer [^{11}C]LMEster at each time point in 60 min of entire scan time.

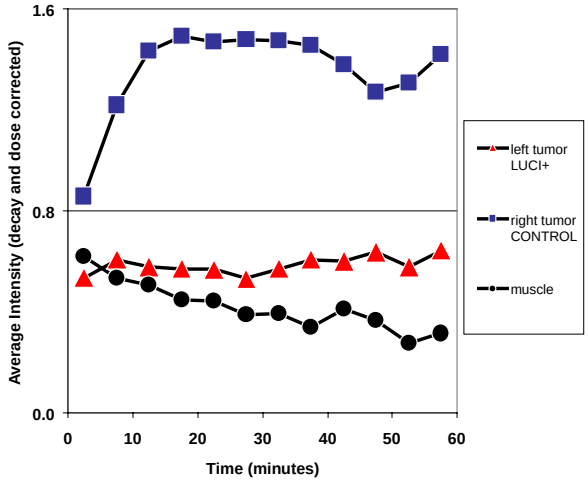


Figure 4. Average left tumor (luciferase), right tumor (control), and muscle intensity versus time from injection of the tracer [^{11}C]LMEther at each time point in 60 min of entire scan time.

The left tumor/muscle (T/M) and right tumor/muscle ratios of the tracers [^{11}C]LMEster and [^{11}C]LMEther in 2 tumor mice are shown in Figure 5. The left tumor/muscle and right tumor/muscle ratios were 1.3 ± 1.8 and 2.1 ± 0.9 for [^{11}C]LMEster. A *t* test comparing left tumor/muscle ratio and right tumor/muscle ratio of [^{11}C]LMEster gave a *p* value of 0.646 which did not denote the presence of a statistically significant difference. The left tumor/muscle and right tumor/muscle ratios were 1.3 ± 0.3 and 2.7 ± 0.0 for [^{11}C]LMEther. A *t* test comparing left tumor/muscle ratio and right

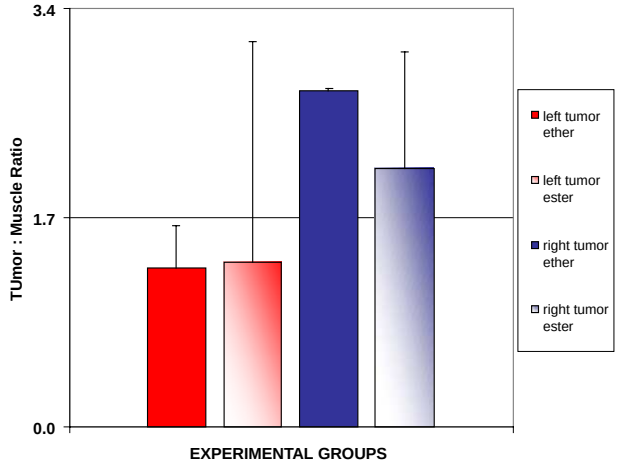


Figure 5. The left tumor/muscle and right tumor/muscle ratios of tracers [^{11}C]LMEster and [^{11}C]LMEther in 2 tumor mice.

tumor/muscle ratio of [^{11}C]LMEther gave a p value of 0.027 which did denote the presence of a statistically significant difference. Likewise, a t test comparing left tumor/muscle ratio difference between [^{11}C]LMEster and [^{11}C]LMEther gave a p value of 0.456, and a t test comparing right tumor/muscle ratio difference between [^{11}C]LMEster and [^{11}C]LMEther gave a p value of 0.447, which also did not denote the presence of a statistically significant difference. Based on Figure 5, there is no significant difference in the T/M ratios between the ether and ester. However, it is important to note that the standard deviations (SD) for the ether are better than those for the ester. Moreover, the contrast of the left and right tumors seems to be better for the ether. Therefore, we can conclude the difference is due to the binding affinity of two radiotracers, rather than simply distribution characteristics of two radiotracers. The available data show [^{11}C]LMEther appeared to have better tumor uptake than [^{11}C]LMEster, which is consistent with their bioluminescence mechanism, since the carboxylic acid group of D-luciferin is the active position to react with ATP and magnesium ion, and this is not a case for the hydroxyl group of D-luciferin. A more likely explanation for this in vivo imaging result is difference in metabolism between [^{11}C]LMEther and [^{11}C]LMEster. The potential metabolism mechanism[26] with O-demethylation at ester position for [^{11}C]LMEster is easier than the potential metabolism mechanism with O-demethylation at ether position for [^{11}C]LMEther, as indicated in Figure 6, therefore, [^{11}C]LMEster appears to have a fast washout without longer retention in the tumor than [^{11}C]LMEther. It is also possible that methylation increases the lipophilicity of the resulting radiotracers. Octanol–water partition coefficient ($\log P$) of the products [^{11}C]LMEster ($\log P$ 2.23) and [^{11}C]LMEther ($\log P$ 1.16) as compared to that of D-luciferin ($\log P$ −0.28) was measured by the HPLC method,[27] which showed [^{11}C]LMEster is more lipophilic to be washed out than [^{11}C]LMEther. This piece of lipophilicity information in addition to the cell uptake data may also partially account for the poor tumor activity accumulation in vivo because of rapid washout of the tracer.

Since the rodent PET imaging afforded disappointing results, to compare different imaging modalities, the optical imaging[28] was performed using a Berthold LB981 NightOwl system consisting of a highly sensitive Peltier CCD camera. NOD/SCID SKOV-3x tumor-bearing mouse was administered with an intraperitoneal injection of 15 μg/kg of unlabeled substrate D-luciferin, and then immediately placed in the NightOWL light-tight chamber and a bioluminescent image was acquired every 2 min for up to 40 min (luminescence dynamics represent luciferin kinematics). For the optical imaging of radiotracers, NOD/SCID SKOV-3x tumor-bearing mouse was administered with an intravenous injection of the tracer [^{11}C]LMEster or [^{11}C]LMEther and scanned with IndyPET-II. After 60 min of dynamic PET imaging, no additional unlabeled D-luciferin added, the bioluminescence image was acquired with CCD camera from the mouse every 5 min up to 25 min. The optical images of unlabeled substrate D-luciferin, and radiotracers [^{11}C]LMEster and [^{11}C]LMEther in 2 tumor mice are shown in Figure 7. Optical imaging with D-luciferin, [^{11}C]LMEster, and [^{11}C]LMEther provided tumor luciferase images within a few minutes of photon counting. The optical imaging shows a more positive result than with PET imaging, which is against our original purpose. Therefore, PET imaging luciferase with [^{11}C]LMEster or [^{11}C]LMEther is likely an unsuccessful attempt, but it will alert other investigators to the need to consider alternative approach in designing molecules for PET imaging luciferase and help direct future research. In comparison of bioluminescence images with PET images, the outcome of PET imaging shows that there is no selective radiolabeled substrate distribution to the luciferase-containing tumors; therefore, we can

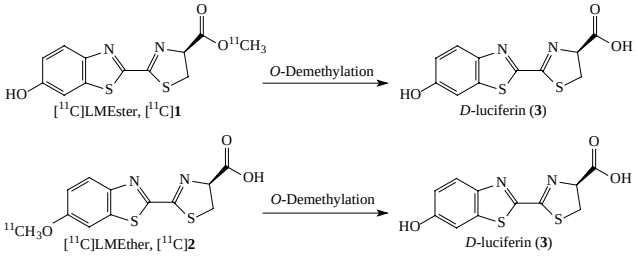
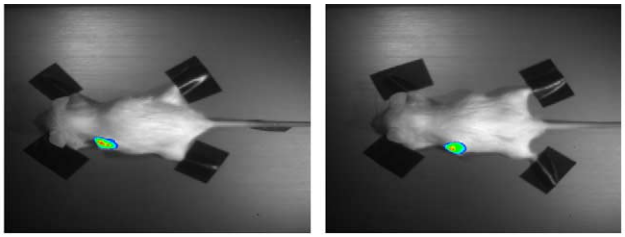
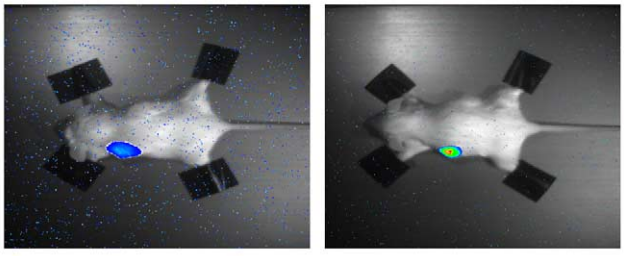


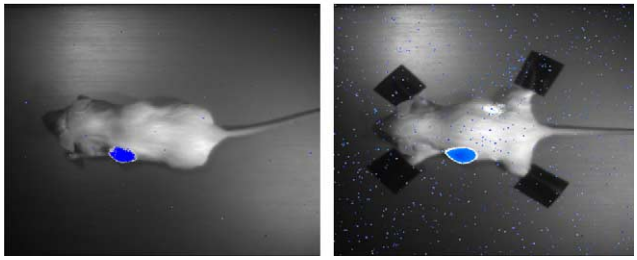
Figure 6. Potential metabolic pathways of tracers [^{11}C]LMEster and [^{11}C]LMEther.



D-Luciferin optical images in tumor mice 1 and 2 (from left to right)



[^{11}C]LMEster optical images in tumor mice 1 and 2 (from left to right)



[^{11}C]LMEster optical images in tumor mice 1 and 2 (from left to right)

Figure 7. Bioluminescence images of unlabeled substrate D-luciferin and radiotracers [^{11}C]LMEster and [^{11}C]LMEther in 2 tumor mice.

conclude that the bioluminescence images are solely or largely the result of selective radiolabeled substrate consumption.

The experimental details for compound **1** and new tracers [^{11}C]**1** and [^{11}C]**2**, tumor xenografts of living mice and optical imaging, are given.[29] The PET imaging, and image registration and fusion were performed using a modification of the literature procedure.[30]

In summary, new C-11 labeled luciferase substrates [^{11}C]LMEster and [^{11}C]LMEther have been well synthesized. An animal model in mice bearing control and luciferase-expressing tumor xenografts has been well developed. Initial PET studies demonstrate a lower retention of the C-11 label at 45 min post-injection in luciferase expression SKOV-3x tumor and no selective radiolabeled substrate distribution to the luciferase-containing tumors. Optical imaging with unlabeled substrate D-luciferin, and radiotracers [^{11}C]LMEster and [^{11}C]LMEther gave tumor luciferase images and serves as a good reference for PET approach. The comparison of bioluminescence images between unlabeled substrate and radiotracers, and the comparison of bioluminescence images with PET images of radiotracers confirm the newly developed C-11 tracers do not work for PET imaging, and the bioluminescence images of C-11 tracers are the result of selective radiolabeled substrate consumption.

Acknowledgments

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29. (a) Compound **1**: To a 100 mL two-necked flask equipped with a magnetic stir bar were added D-luciferin potassium salt **3** (0.50 g, 1.58 mmol) and hexamethylphosphoramide (HMPA, 20 mL). CH_3OTf (0.25 mL, 2.28 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 24 h. The product was extracted with EtOAc, washed with brine, dried over $MgSO_4$, and concentrated to give a yellowish residue. The residue was subjected to column chromatography eluted with 2:1 hexane/EtOAc to give compound **1** (0.29 g, 63%) as a white solid, mp 198–200 °C, $R_f = 0.20$ (2:1 hexane/EtOAc). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.23 (s, 1H, OH, D_2O exchangeable), 7.95 (d, 1H, $J = 8.83$ Hz, phenyl), 7.44 (t, 1H, $J = 2.21$ Hz, phenyl), 7.06 (d of t, 1H, $J_1 = 2.20$ Hz, $J_2 = 8.83$ Hz, phenyl), 5.51 (d of d, 1H, $J_1 = 8.09$ Hz, $J_2 = 9.93$ Hz, C*H*COOCH$_3$, D_2O exchangeable), 3.74 (s, 3H, CH_3), 3.64–3.89 (m, 2H, CH_2). LRMS (*m*/*z*): 149 (100%), 294 (M^+, 4.7%). HRMS (*m*/*z*): calcd for $C_{12}H_{10}N_2O_3S_2$ 294.0133. Found: 294.0130; (b) Tracer [^{11}C]**1**: D-Luciferin (**3**, 0.6–1.0 mg) was dissolved in

CH_3CN (500 μL). To this solution was added tetrabutylammonium hydroxide (TBAH) (5 μL, 1 M solution in methanol). The mixture was transferred to a small volume, three-necked reaction tube. [^{11}C]CH_3OTf was passed into the air-cooled reaction tube at −15 to −20 °C, which was generated by a Venturi cooling device powered with 100 psi compressed air, until radioactivity reached a maximum (~3 min), then the reaction tube was heated at 70–80 °C for 2 min. The contents of the reaction tube were diluted with $NaHCO_3$ (1 mL, 0.1 M). This solution was passed onto a C-18 cartridge by gas pressure. The cartridge was washed with H_2O (2 × 3 mL), and the aqueous washing was discarded. The product was eluted from the column with EtOH (2 × 3 mL) and then passed onto a rotatory evaporator. The solvent was removed by evaporation under high vacuum. The labeled product [^{11}C]**1** was formulated with saline (1–3 mL), sterile-filtered through a sterile vented Millex-GS 0.22 μm cellulose acetate membrane, and collected into a sterile vial. Total radioactivity was assayed and total volume was noted. The overall synthesis time was 15–20 min. The decay corrected yield, from $^{11}CO_2$, was 40–55%, and the radiochemical purity was >95% measured by analytical HPLC; (c) Tracer [^{11}C]**2**: the LMEster (**1**, 0.6–1.0 mg) was dissolved in CH_3CN (500 μL). To this solution was added TBAH (5 μL, 1 M solution in methanol). The mixture was transferred to a small volume, three-necked reaction tube. [^{11}C]CH_3OTf was passed into the air-cooled reaction tube at −15 to −20 °C, which was generated by a Venturi cooling device powered with 100 psi compressed air, until radioactivity reached a maximum (~3 min), then the reaction tube was heated at 70–80 °C for 2 min. The contents of the reaction tube were diluted with $NaHCO_3$ (1 mL, 0.1 M). This solution was passed onto a C-18 cartridge by gas pressure. The cartridge was washed with H_2O (2 × 3 mL), and the aqueous washing was discarded. The product was eluted from the column with EtOH (2 × 3 mL) and then passed onto a rotatory evaporator. The solvent was removed by evaporation under high vacuum to give a residue [^{11}C]**4**. This residue was added with 1 M HCl (0.6 mL) and MeOH (1 mL), and heated for 5 min. The labeled product [^{11}C]**2** mixture was evaporated under high vacuum to give the pure product [^{11}C]**2**, which was formulated with saline (1–3 mL), sterile-filtered through a sterile vented Millex-GS 0.22 μm cellulose acetate membrane, and collected into a sterile vial. Total radioactivity was assayed and total volume was noted. The overall synthesis time was ~20 min. The decay corrected yield, from $^{11}CO_2$, was 25–35%, and the radiochemical purity was >93% measured by analytical HPLC; (d) Tumor xenografts of living mice: all animal experiments were performed under a protocol approved by the Indiana University Institutional Animal Care and Use Committee. The LucSH cDNA was purchased from InvivoGen (San Diego, CA) and is a fusion between the luciferase and zeocin resistance genes. Both genes have been modified and contain no CpG. The SF91–LUC–EGFP vector co-expressed LucSH and EGFP was pseudotyped with the GALV envelope using the PG13 stable producer line. Tumor cells were transduced and either selected in zeocin or sorted for EGFP+ cells by flow cytometry. The control SKOV-3x tumor and luciferase expression SKOV-3x tumor were implanted to the NOD/SCID mice at the right-hand side and left-hand side of the mouse body, respectively. Tumors were allowed to develop to 171–299 mm^3 for SKOV-3x luciferase tumor and 236–536 mm^3 for SKOV-3x control tumor in 18 days; (e) Optical imaging: the optical imaging was performed using a Berthold LB981 NightOwl system consisting of a highly sensitive Peltier cooled charge-coupled device (CCD) camera. NOD/SCID SKOV-3x tumor-bearing mouse was sedated with 0.2 mg/kg acepromazine (im) and torbugesic (im) prior to imaging. After an intraperitoneal injection of 15 μg/kg of cold substrate D-luciferin, the mouse was immediately placed in the NightOWL light-tight chamber and a bioluminescent image was acquired every 2 min for up to 40 min (luminescence dynamics represent luciferin kinematics). A colorized luminescence intensity image was overlaid onto a black and white photograph image (25 μs white-light exposure). A 10% threshold relative to maximum photon flux was set. Integrated photon flux and tumor surface area are plotted. Images with maximum flat response were used to determine elliptical properties of the tumor. Assuming a symmetric ellipsoidal volume, volume estimates were determined. The bioluminescence images of radiotracers [^{11}C]LMEster and [^{11}C]LMEther were acquired with CCD camera from the tumor mouse after IndyPET-II scans without additional intraperitoneal injection of unlabeled substrate D-luciferin.

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