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Evaluation of NanoLuc substrates for bioluminescence imaging of transferred cells in mice

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ABSTRACT

NanoLuc luciferase recently gained popularity due to its small size and superior bioluminescence performance. For *in vivo* imaging applications, NanoLuc has been limited by its substrate furimazine, which has low solubility and bioavailability. Herein, we compared the performances of recently reported NanoLuc luciferase substrates for *in vivo* imaging in mice. Two substrates with improved aqueous solubility, hydrofurimazine and fluorofurimazine, were evaluated along with three stabilized *O*-acetylated furimazine analogues, the hikarazines. All 5 analogues, when tested *in vitro*, displayed greater signal intensity and reaction duration, in comparison to the standard NanoLuc substrate, furimazine. The two best-performing analogues from the *in vitro* study were selected for further *in vivo* testing. The NanoLuc/fluorofurimazine pair demonstrated the highest bioluminescence intensity, post intravenous administration. It was found to be around 9-fold brighter compared to the NanoLuc/furimazine and 11-fold more intense than the NanoLuc/hikarazine-003 pair, with an average of 3-fold higher light emission when the substrate was injected intraperitoneally, in a subcutaneous model. Excitingly, despite the fact that NanoLuc/fluorofurimazine emits mostly blue light, we prove that cells trapped in mice lungs vasculature could be visualised *via* the NanoLuc/fluorofurimazine pair and compare the results to the AkaLuc/AkaLumine system. Therefore, among the tested analogues, fluorofurimazine enables higher substrate loading and improved optical imaging sensitivity in small animals, upgrading the use of NanoLuc derived bioluminescent systems for deep tissue imaging.

1. Introduction

Bioluminescence imaging (BLI) is based on a biochemical reaction that is dependent on the oxidation of a luminophore containing substrate (luciferin) by luciferase enzymes with light emission as a result. Luciferase enzymes and their substrates are extensively used as effective, non-invasive screening tools in diverse research fields and have become a prominent method for live-cell visualization. Improving the performance of luciferase enzymes, substrates and their mutants for BLI is an important part in improving the tool box available for life science research [1].

Several thousand bioluminescent species are represented by ~700 genera among which 90% originate from marine organisms [2,3]. Luciferases from marine luminous organisms primarily utilise as substrates, coelenterazine [2], varguline [3] or analogues of these two. Coelenterazine (CTZ) is best-known as the substrate for luciferases derived from the sea pansy *Renilla reniformis (RLuc)*, the luminous shrimp *Oplophorus gracilirostris* and the copepod *Gaussia princeps (GLuc)*, yielding light with a fast enzymatic turnover and an emission maxima in the blue region (450–485 nm) of the visible spectrum.

NanoLuc (NLuc) is one of the smallest luciferases in size (19 kDa) and an important addition to the marine BLI-toolset [4]. Its small size,

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luminescence superiority and stability easily replaced other luciferases where increased sensitivity, fast response dynamics and low background auto-luminescence are vital [5]. The NLuc system is coupled with an optimized CTZ analogue, the 2-furanylmethyl-deoxycoelenterazine, known as furimazine, which when paired with NLuc gives ~2.5 million-fold brighter luminescence in mammalian cells and ~150-fold greater specific activity than firefly (*Photinus pyralis*) or *Renilla* luciferases [4].

Furimazine and its analogues are generally lipophilic and their solubility in aqueous solutions has limited the maximum injectable dose into small animals (*e.g.* 1.3 μmol in a polyethylene glycol (PEG) [6] based buffer and 0.016 μmol in Phosphate Buffered Saline (PBS) [7]. Therefore, furimazine analogues with improved solubility and bioavailability are needed to improve NanoLuc sensitivity for *in vivo* imaging [8]. Moreover, the NanoLuc/furimazine system mainly emits blue light, further limiting the sensitivity of detection in deep tissue.

Many synthetic analogues of CTZ have been reported in recent years, mostly focusing on the modification of the C-2, C-5, C-6 and C-8 substituents of the imidazopyrazinone core [4,9], with the most effort on modifying the C-2 and C-6 position [5–7]. The novel analogues were mostly optimized in order to improve *in vivo* imaging with NanoLuc, leading to better compound stability and more efficient light penetration through tissues [5–7]. A chemical library of 135 CTZ-analogues, the imidazo[1,2-*a*]pyrazin-3-one, have been recently synthetized as stabilized *O*-acetylated precursors (hikarazines) with an original synthesis route [10,11]. Some examples show robust bioluminescent properties in terms of signal intensity and duration of the reaction. Particular examples turned out to be biochemically more efficient than furimazine with up to 2.5 increased light intensity and signal stability lasting up to 2 h [11]. Novel furimazine analogues, with increased aqueous solubility, were recently developed and named hydrofurimazine (HFz) and fluorofurimazine (FFz) [12]. These analogues enable *in vivo* applications by allowing the delivery of higher substrate doses *via* single intraperitoneal injection in mice leading to extended light emission *in vivo* for NanoLuc [13].

In the current work we investigated the BLI properties of novel furimazines (HFz and FFz) [11] and 3 different hikarazines (hikarazine-001, hikarazine-003 and hikarazine-097) [14–16] by evaluating their *in vivo* performance as substrates for imaging with NanoLuc, as the reference marine luciferase since recently they have demonstrated to be better suited for *in cellulo* and *in vivo* NLuc based imaging [14–16].

Although the above mentioned NanoLuc substrates have been reported to perform well *in vivo*, no direct comparison has been performed yet. In the present study, we first evaluated 5 recently reported analogues *in vitro*, through controlled conditions, using cells expressing a known amount of NanoLuc reporter, following with an *in vivo* comparison in a subcutaneous model. Secondly, since BLI in deep tissue is mostly dependent on the amount of generated photons and the emission wavelength, we tested the best performing NLuc/substrate pairing (NLuc/fluorofurimazine) in deep tissue, by imaging transferred bioluminescent cells that get entrapped in mice lungs, and compared it to the near-infrared AkaLuc/AkaLumine system [17], recently described by Iwano et al.

2. Materials & Methods

2.1. Cell Propagation for Life Cell Assays

Human embryonic kidney (HEK-293T) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured in T-175 flasks (Thermo Fisher Scientific) until reaching confluence; growth media was aspirated and cells were washed with PBS (Sigma-Aldrich). The PBS was aspirated and the cells were removed from the flask by the addition of 3 mL of TrypLE™Express Trypsin (Life Technologies) and were incubated at 37 °C. Cells were centrifuged at 1500 RPM, washed, re-suspended in 5 mL of fresh growth media, and then counted using a BioRad TC20 cell counter. Cells were diluted to a desired final concentration and were plated in 96 well plates (Greiner Cell Star®) in total volume of 100 μL at 25000 cells/well. The culture was incubated at 37 °C with 5% CO_2.

2.2. Lentiviral Production

The plasmids pCDH-EF1-NanoLuc-T2A-copGFP and pCDH-EF1-AkaLuc-T2A-copGFP were engineered by NanoLuc or AkaLuc luciferase gene insertion into the vector backbone pCDH-EF1-T2A-copGFP. The inserted NanoLuc/AkaLuc luciferase genes were amplified with specific primers from pCDH-EF1-NanoLuc plasmid (Promega)/pcDNA3 Venus-AkaLuc plasmid (Riken BRC Repository) without a stop codon using BamHI and NotI sites into the cut vector: pCDH-EF1-MCS-T2A-copGFP. The pCDH-EF1-NanoLuc-copGFP/pCDH-EF1-AkaLuc-T2A-copGFP lentivirus were constructed using methods already described earlier in literature [18,19]. Virus production and cell transduction were performed under appropriate biosafety level conditions (ML-II) in accordance with the National Biosafety Guidelines and Regulations for Research on Genetically Modified Organisms. Procedures and protocols were reviewed and approved by the EMC Biosafety Committee (GMO permit 99-163).

Lentiviral particles were produced by transfection of HEK-293T packaging cells with three packaging plasmids (pCMV-VSVG, pMDLg-RRE (gag-pol), pRSV-REV) (Addgene) and the lentiviral vector plasmid using PEI transfection reagent (1 mg/mL)/μg DNA. The supernatant, containing lentiviral particles was collected after 48 h and 72 h. Viral quantification was performed using the standard antigen-capture HIV p24 ELISA (ZeptoMetrix).

2.3. Generation of Luciferase-Expressing HEK-293T Cell Lines

HEK-293T cells were grown in culture dishes to 50% confluency in culture medium and were infected with a lenti-viral stock, resulting in NanoLuc or AkaLuc expression, depending on the used lentivirus. Cells were transduced with MOI 1 of pCDH-EF1-NanoLuc-T2A-copGFP/pCDH-EF1-AkaLuc-T2A-copGFP lentivirus plus with polybrine (hexametride bromide) (Sigma-Aldrich) at the final concentration of 8 μg/mL. Cells were sorted for GFP expression using FACS (BD-FACS AriaIII, BD Biosciences). Transgene expression was confirmed by the presence of the green fluorescent protein copGFP (excitation/emission maximum = 475/509 nm). Stably transduced cells (HEK-293T-EF1-NanoLuc-T2A-copGFP/HEK293T-EF1-AkaLuc-T2A-copGFP) were further cultured and expanded and have been used later for all experiments regarding measurement of photon production when using different furimazine analogues.

2.4. In Vitro Cell-Based Imaging

HEK-293T-NanoLuc cells were seeded in a 96-well black plate (Greiner Cell Star®) at a density of 25 000 cells per well. Post 24 h, cells were washed in PBS and imaged at the IVIS Imager (PerkinElmer) after addition of 6 different substrates profiled within this study; furimazine, hydrofurimazine (HFz), fluorofurimazine (FFz), all three provided by Promega and the hikarazine-001, hikarazine-003 and hikarazine-097 provided by Institute Pasteur, Paris, France. The hikarazines (hikarazine-001, hikarazine-003, hikarazine-097) were dissolved (1 mg) in a mixture of 0,2 mL DMSO (Sigma-Aldrich) and then diluted in a solution of 0,3 mL of acidic ethanol made from addition of 37% hydrochloric acid (Sigma-Aldrich), 100 μL on 12 mL of 100% ethanol (Sigma-Aldrich) to give a stock solution of 1 mg/mL which was then incubated at 50 °C for 2 h. Furimazine, hydrofurimazine (HFz) and flurofurimazine (FFz) were dissolved in 100% ethanol to give a 5 mM stock solution. All of the substrates were further diluted in DMEM medium to a final concentration of 10 μM (100 μL/well). Bioluminescence signal from wells was

measured at the IVIS (Perkin Elmer) spectrum system every 5 min after substrate addition. All *in vitro* cell-based measurements were acquired after 1 min at 37 °C using a 30 s acquisition time with an open filter. Experiments were performed in triplicates and were repeated three times. Data was analyzed using Living Image 4.3 software (Perkin Elmer) by applying the appropriate region of interest (ROI).

As for the once-cell assay; HEK-293T-NanoLuc cells were plated in a black 96-well plate in seven dilutions starting at a density of approximately 100 cells per well as the starting cell-concentration. Further cell-dilutions were carried out with a ½ ratio; (50, 25, 12, 6, 3, 1, 0) cells per well. Luminescence images were acquired with the GloMax® Microplate Reader (Promega) immediately after substrates addition; furimazine, hydrofurimazine (HFz), fluorofurimazine (FFz), hikarazine-001, hikarazine-003 and hikarazine-097 with a final concentration of each substrate of 10 μM. For measurement of total luminescence, we used 1 s of integration time. Measurements were performed at room temperature 18–20 °C. Experiments were performed in triplicates and were repeated three times. All data were plotted using GraphPad Prism.

2.5. Substrate Formulation for Use in Whole-Animal Imaging

The hikarazines (hikarazine-001, hikarazine-003, hikarazine-097) (Institute Pasteur, Paris, France) were dissolved (1 mg) in a mixture of 0.2 mL DMSO (Sigma-Aldrich) and then diluted in a solution of 0.3 mL of acidic ethanol made from addition of 37% hydrochloric acid (Sigma-Aldrich), 100 μL on 12 mL of 100% ethanol (Sigma) to give a stock solution of 1 mg/mL which was then incubated at 50 °C for 2 h. The stock solution was then dissolved in PBS at a final concentration of 333 nmol in 100 μL. Furimazine, hydrofurimazine (HFz) and fluorofurimazine (FFz) (Promega), powder formulations for intravenous administration, were dissolved in 100% ethanol to give a 5 mM stock solution. The stock solution was then dissolved in PBS to a final dose of 333 nmol in 100 μL. Stock aliquots of the poloxamer-407 HFz and FFz, for the intraperitoneal administration, were prepared by the addition of 480 μL of PBS to the vial and vortexed to create a plain solution resulting in a single injection dose of 12 mg poloxamer-407 and 4.3 μmol HFz and FFz, and 1.3 μmol (145 μL).

AkaLumine (Sigma-Aldrich) was dissolved in PBS to a concentration of 33 mM (around 3 μmol/100 μL) for i.p. injection. These doses were chosen considering maximum solubility and tolerability in mice and maximum attainable signal based on previous findings.

2.6. In Vivo Bioluminescence Imaging of Subcutaneously Implanted NanoLuc Expressing Cells

Animal experiments were approved by the Bioethics Committee of Erasmus MC, Rotterdam, The Netherlands and performed in accordance with national guidelines and regulations established by the Dutch Experiments on Animal Act (WoD) and by the European Directive on the Protection of Animals used for scientific purpose (2010/63/EU). BALB/c nude (males) were obtained from Charles River Laboratory (The Netherlands). All mice aged 8 weeks were provided access to food and water *ad libitum* and were hosted in the animal facility at the Erasmus MC, Rotterdam, The Netherlands.

For the background assessment mice (n = 3 mice per tested analogue) were injected intraperitoneal (i.p.) with the following analogues; 4.3 μmol (in 480 μL PBS) and 1.3 μmol (in 145 μL PBS) of FFz; 333 nmol of furimazine and hikarazine-003, both in 100 μL of PBS and 3 μmol of AkaLumine (Sigma-Aldrich) in 100 μL of PBS. Mice were anesthetized with isoflurane (1.5%) and imaged 10 min post substrate administration at the IVIS Spectrum (PerkinElmer). Supine and prone BLI images were acquired at the IVIS, exposure time 1 min.

For the subcutaneous skin model experiments, 8–10 week old BALB/c nude (males) mice received a subcutaneous injection of 1×10^6 HEK-293T-NanoLuc which were prepared in PBS (Sigma-Aldrich) and matrigel (Corning) solution (50:50 ratio) in a final injectable volume of 50 μL. Mice received an injection of 100 μL of the different substrate intravenously ($n = 6$ mice per group) or intraperitoneally (480 μL) ($n = 3$ mice per group) right after implantation of cells. The size of the group was determined using power analysis. Mice were kept under isoflurane anaesthesia (1.5%) and a series of images were taken using an IVIS Spectrum with open filter binning = medium, field of view = 12.9 × 12.9 cm, f/stop = 1 and 1 s exposure time every 5 min for half an hour. Data analysis was performed by drawing ROIs in the images taken at the peak of bioluminescence emission.

2.7. In Vivo BLI Comparison of HEK-293T Cells Trapped in the Mouse Lung (NLuc/Fluorofurimazine vs. AkaLuc/AkaLumine)

BALB/c nude (males) were used for the experimental purposes. Post cell selection at the flow-cytometry, either HEK-293T-AkaLuc or HEK-293T-NanoLuc cells were re-suspended and aliquoted (500000 cells/100 μL) each, and injected into the tail vein of mice.

10 to 15 min after the cell infusion, the mice were anesthetized with 1.5% isoflurane for induction and administered *via* intraperitoneal injection with the respective substrate (100 μL of 3 μmol AkaLumine, 145 μL of 1.3 μmol fluorofurimazine (FFz). 10 min post substrate administration, dorsal BLI images were taken with 1,5% isoflurane and using the IVIS Spectrum (PerkinElmer) with following acquisition conditions: open for total bioluminescence, exposure time = 1 min, binning = medium: 4, field of view = 12.5 × 12.5 cm, and f/stop = 1. Analysis of BLI images was performed with Living Image 4.3 software (PerkinElmer).

2.8. Statistical Analysis

For experiments where more than two groups were compared, one-way ANOVA, followed by Tukey's *t*-test was used to determine significant differences among treated groups.

3. Results

3.1. In Vitro Performance Comparison of Novel Analogues Paired with NanoLuc Luciferase

Bioluminescence properties of the chosen furimazine analogues (Fig. 1), profiled with NLuc were assessed in live HEK-293T cells that were stably expressing NLuc luciferase in order to determine the best-performing NLuc/analogue pairs for *in vivo* testing. Cells were treated with substrates at a final concentration of 10 μM each and imaged in the IVIS imager at 5 min intervals after substrate administration for half an hour (Fig. 2a).

The imaging results in live cells showed brighter signals with the hikarazines (hikarazine-001, hikarazine-003 and hikarazine-097) than furimazine and its analogues hydrofurimazine (HFz) and fluorofurimazine (FFz) (Fig. 2b). We found that peak light emission was highest with hikarazine-003, followed by hikarazine-001, hikarazine-097 and fluorofurimazine. The signal of all tested analogues proved to be extended and stable over the 30 min imaging duration.

We evaluated the correlation between reporter signal and number of cells, by plating a decreasing number of cells, ranging from 100 to 1 cell per well. Linear correlation was found and detection of single cells was achieved with all tested analogues. Again, the hikarazines showed to be brighter: hikarazine-003 = 2.75e+004 rlu/cell; hikarazine-001 = 2.48e+004 rlu/cell; hikarazine-097 = 2.16e+004 rlu/cell to the novel furimazine analogues and to furimazine itself: furimazine = 1.42e+004 rlu/cell; hydrofurimazine = 1.07e+004 rlu/cell and fluorofurimazine = 2.32e+004 rlu/cell (Fig. 2c).

Since the photon emission was comparable among the 3 tested hikarazines, we selected the brightest analogue (hikarazine-003) for further *in vivo* evaluation among with fluorofurimazine (FFz).

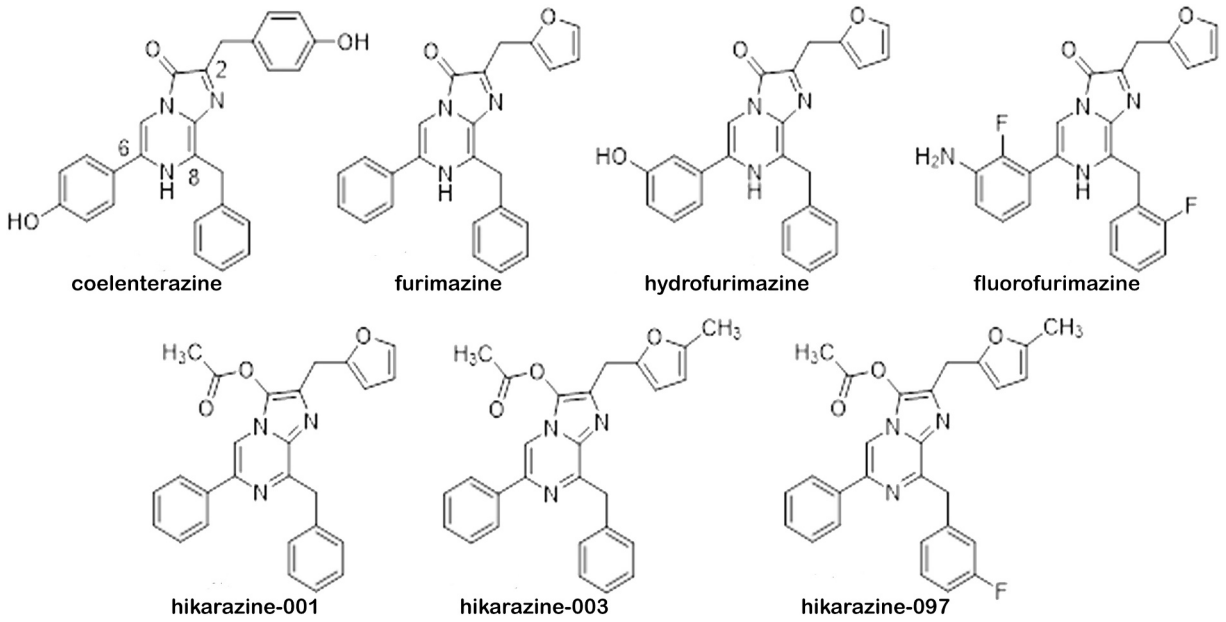

Fig. 1. Chemical structures of furimazine analogues in this article; hikarazine-001, hikarazine-003, hikarazine-097, hydrofurimazine (HFz), fluorofurimazine (FFz) and furimazine.

3.2. *In Vivo Bioluminescence Imaging of Subcutaneously Implanted Cells*

Prior to testing the compounds in mice, we assessed background emission of all substrates, including the AkaLuc substrate, AkaLumine, in nude mice not expressing a luciferase reporter. We administered the substrates intraperitoneally, allowing us to estimate autoluminescence in the absence of reporter enzymes (NanoLuc/AkaLuc). All substrates were administered with the doses recommended by the literature as appropriate for each substrate. Negligible signals were detected with AkaLumine, whereas all tested NLuc analogues gave background signal of around 10^2 ph/s in the abdomen in nude mice (Supplementary Fig. 1).

Given their intense *in vitro* brightness, we selected FFz and hikarazine-003 for further *in vivo* evaluation. Each substrate was administered *via* intravenous (i.v.) and intraperitoneal (i.p.) injection in mice. Around 1×10^6 HEK-293T cells were transferred in nude mice and injected subcutaneously in the lower flank, stably expressing NanoLuc luciferase.

For the i.v. administration route, the images were acquired after tail vein injection of equimolar substrate doses (injection volume 100 µL); fluorofurimazine (333 nmol) (Fig. 3a), furimazine (333 nmol) (Fig. 3b) and hikarazine-003 (333 nmol) (Fig. 3c). The results demonstrated that the NanoLuc/fluorofurimazine pair was significantly brighter (around 8.6 fold) than the NanoLuc/furimazine pair and around 11.62 fold higher than the NanoLuc/hikarazine/003 pair (Fig. 3d) when administered i.v. at equimolar doses. The signal with the NanoLuc/fluorofurimazine pair showed a very high photon emission for the whole 30 min of acquisition, with an average of 3.58E+09 ph/s and an initial luminescence (1 min post injection) of 1.70E+10 (Fig. 3d).

We further evaluated the BLI emission when administering the analogues i.p. We tested the newly formulated fluorofurimazine (FFz) in 3 different ways; with a dose of 4.3 µmol (Fig. 4a) and 333 nmol derived from the solubilised poloxamer-P407 cake formulations [11] (Fig. 4c), and with fluorofurimazine derived stock powder formulation (333 nmol) (Fig. 4b). Hikarazine-003 and furimazine were administered with a final concentration of 333 nmol (Fig. 4d and e).

As shown in Fig. 5 the 4.3 µmol NanoLuc/fluorofurimazine pair showed excellent BLI properties with a very high, sustained signal of an average of 1.22E+10 ph/s, around 11 fold higher than the reference 333 nmol NanoLuc/furimazine pair and 3 fold higher than the 333 nmol NanoLuc/hikarazine-003 combination. The fluorinated FFz (333 nmol; 4.3 µmol) and its powder stock formulation (333 nmol) generated 3-fold brighter signal than hikarazine-003 (Fig. 5).

3.3. *In Vivo Deep Tissue BLI: NLuc/Fluorofurimazine vs. AkaLuc/AkaLumine*

To assess how well NLuc in combination with FFz performs in deep tissue, we evaluated the brightness from HEK-293T cells stably expressing NanoLuc and copGFP, and compared it to photon emission of cells expressing AkaLuc and copGFP. We used GFP to ensure equimolar expression of both bioluminescent enzymes. The luciferase expressing cells were localised in mice lungs after tail vein injection of approximately 5×10^5 HEK-293T expressing either NLuc-copGFP or AkaLuc-copGFP [20,21] (Fig. 6a). A dose of 4.3 µmol FFz gives more light, but was found to cause weight loss and was associated with organ damage in mice: later on, a dose was established which showed no toxicity in mice consisting of 1.3 µmol FFz in P-407 [12], which we implemented in this experiment. Mice were imaged 10 min after cell administration and a kinetic analysis was performed after i.p. injection of substrate (1.3 µmol/145 µL FFz; 3 µmol/100 µL AkaLumine) for 30 min. We were able to detect signals from both luciferase systems with a 1.5-fold higher photon emission arising from the AkaLuc/AkaLumine system (Fig. 6b). For the emission comparison, values at the time point of peak emission were used. The signals were corrected by a factor of 2.3 fold, given the higher-level of GFP expression of HEK-293T-AkaLuc expressing cells, determined prior the imaging session.

4. Discussion

In the present study we tested novel furimazine analogues paired with NanoLuc luciferase to determine the most optimal NLuc/luciferin pair for *in vivo* imaging. We report on photon yields of two novel, furimazine analogues, recently published by Su, Y. et al., named hydrofurimazine (HFz) and fluorofurimazine (FFz) and three different *O*-acetylated furimazine analogue derivates [9], known as 'hikarazines' (hikarazine-001, hikarazine-003 and hikarazine-097) and we confirm that many of these novel analogues improve the efficient brightness of NanoLuc *in vitro* and *in vivo*.

Our aim was to compare the best performing analogues in an *in vivo* setting based on different routes of administration. The comparison of

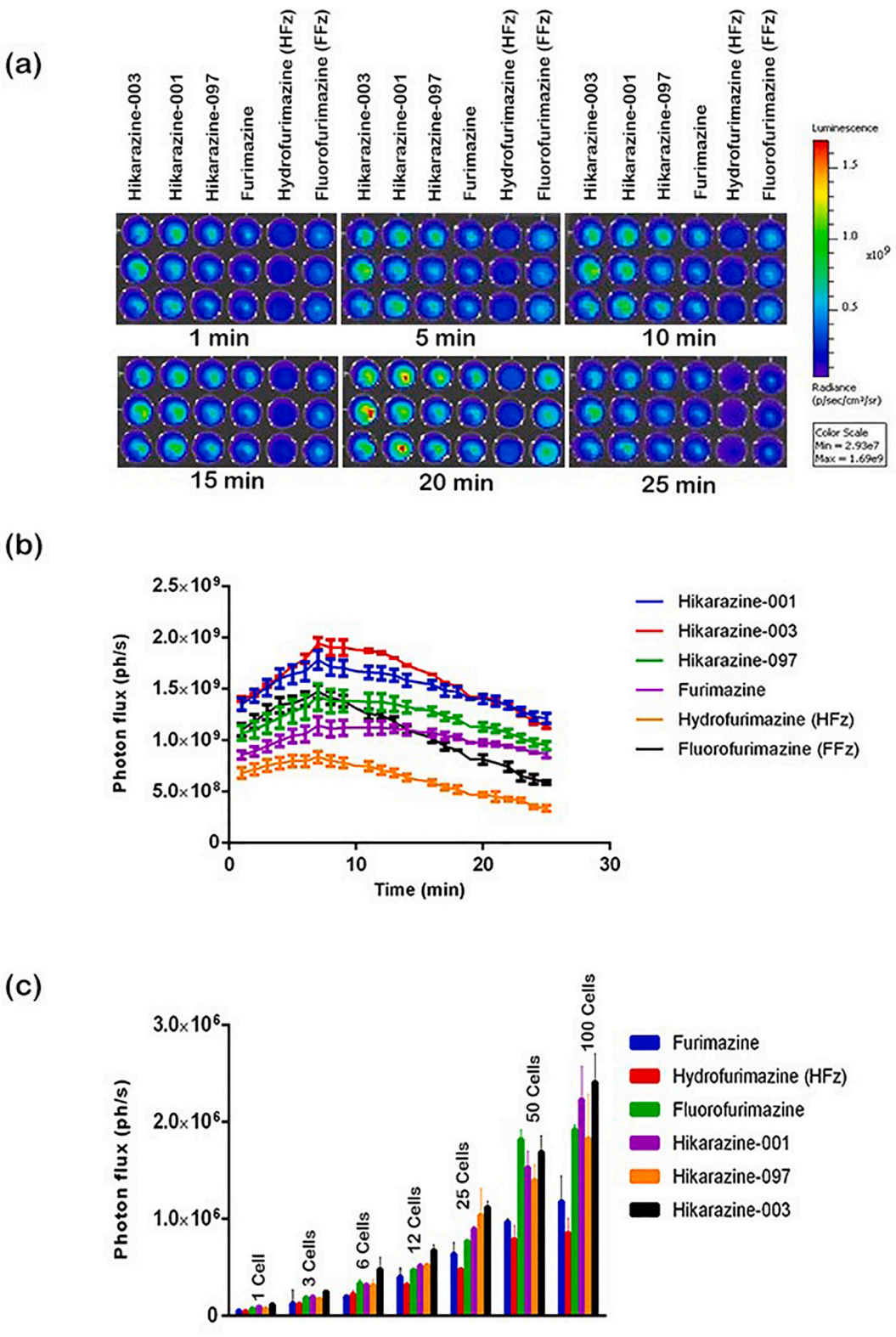

Fig. 2. *In cellulo* performance of novel furimazine analogues in live HEK-293T cells transiently expressing NanoLuc luciferase. (a) Bioluminescence (BLI) imaging of substrates profiled with NanoLuc luciferase expressing cells. (b) Comparison of BLI-intensities of tested furimazine derivatives at a 10 μM concentration. BLI spectra were obtained at the IVIS Imager in triplicates, acquisition time of 1 s. Statistical analysis of triplicates was performed using ONE-Way Anova followed by Tukey's *t*-test (p value < 0.001). (c) Comparison of bioluminescence intensities of the substrates in HEK-293T cells expressing NanoLuc luciferase (NLuc) at various cell-concentrations. Luminescence images were acquired with the GloMax® Microplate Reader (Promega) immediately after substrates addition (10 μM), 1 s integration time.

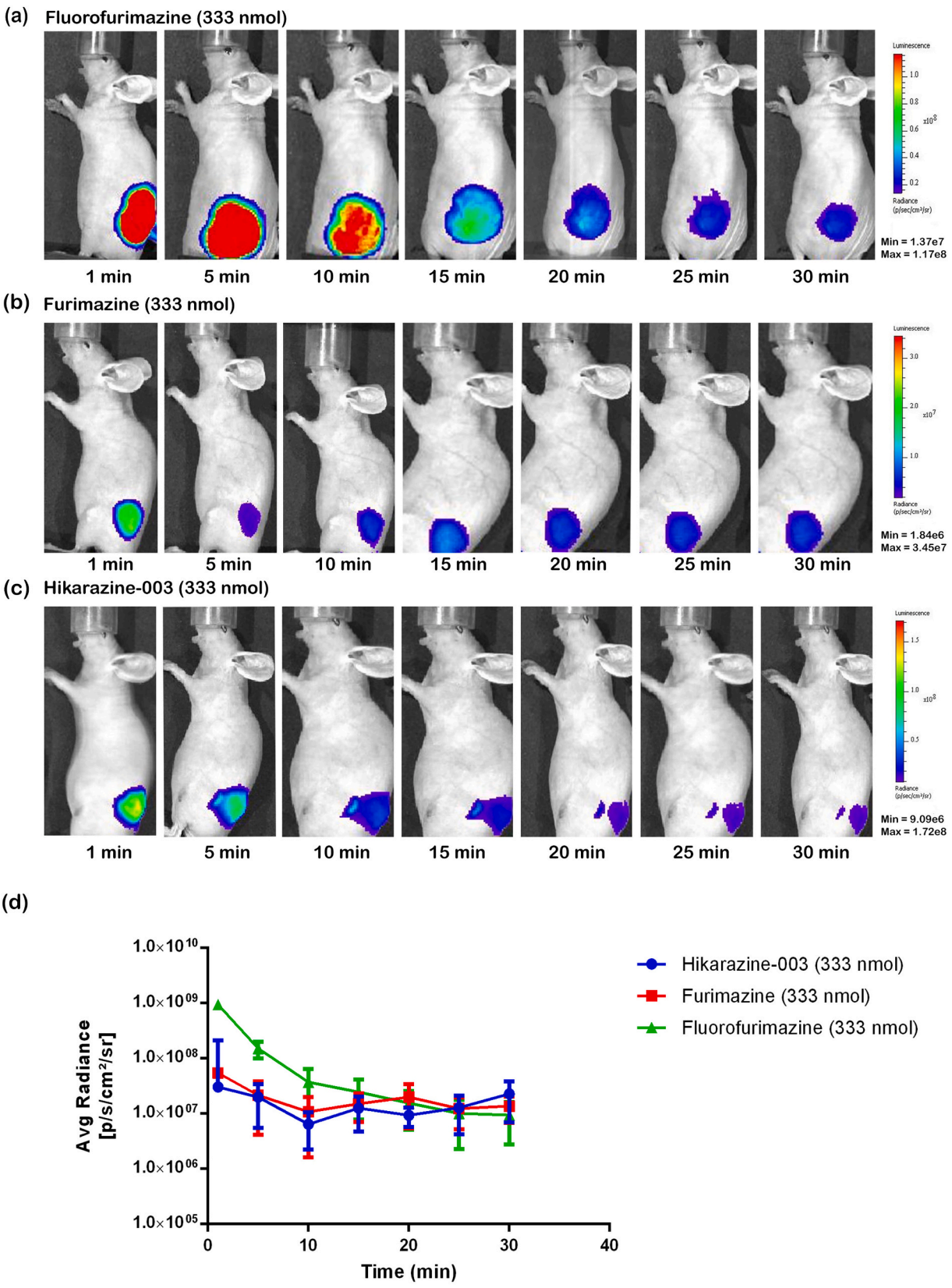

Fig. 3. *In vivo* imaging of fluorofurimazine, hikarazine-003 and furimazine at 333 nmol after intravenous (i.v) administration in a subcutaneous mice model. *In vivo* imaging after (i.v.) administration ($n = 6$) of; (a) fluorofurimazine; (b) furimazine and (c) hikarazine-003. (d) Quantification of total maximum flux of fluorofurimazine, hikarazine-003 and furimazine at 333 nmol after intravenous administration. Spectral data was acquired for 30 min, every 5 min after substrate administration. Imaging data was collected at the IVIS Imager for 30 min using open filters and an exposure time of 1 s.

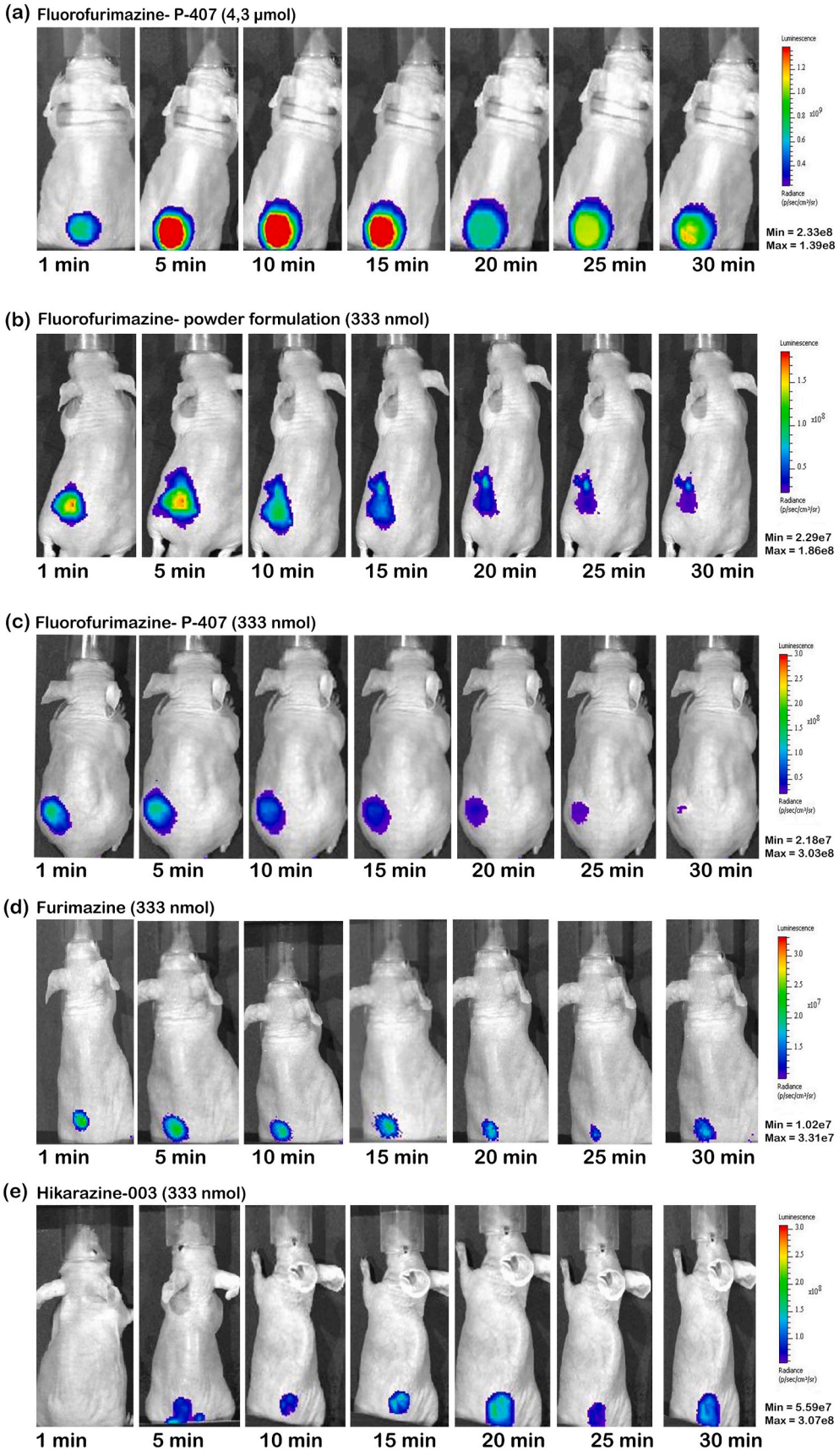

Fig. 4. *In vivo* imaging of fluorofurimazine, hikarazine-003 and furimazine at different concentrations after intraperitoneal (i.p.) administration in a subcutaneous mice model. *In vivo* imaging after i.p. administration of ($n = 3$); (a) fluorofurimazine-P407 formulation (4.3 µmol); (b) fluorofurimazine powder stock formulation (333 nmol); (c) fluorofurimazine-P407 formulation (333 nmol); (d) furimazine (333 nmol) and (e) hikarazine-003 (333 nmol). Spectral data was acquired for 30 min, every 5 min after substrate administration using open filters and an exposure time of 1 s.

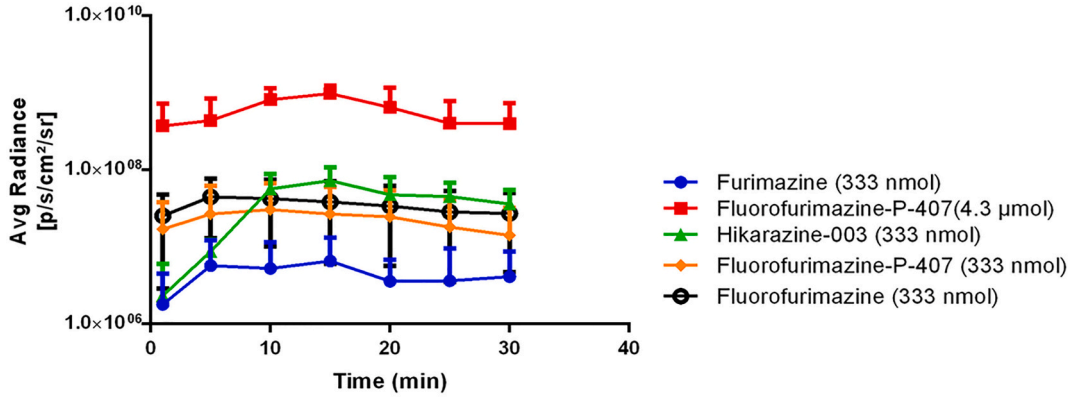

Fig. 5. Signal quantification after intraperitoneal (i.p.) administration of fluorofurimazine, hikarazine-003 and furimazine at different concentrations. Fluorofurimazine (333 nmol and 4.3 µmol)-P407 formulation and FFz (333 nmol) from the powder stock formulation showed significantly higher photon emission *in vivo* than furimazne and hikarazine-003 (p value < 0.0001).

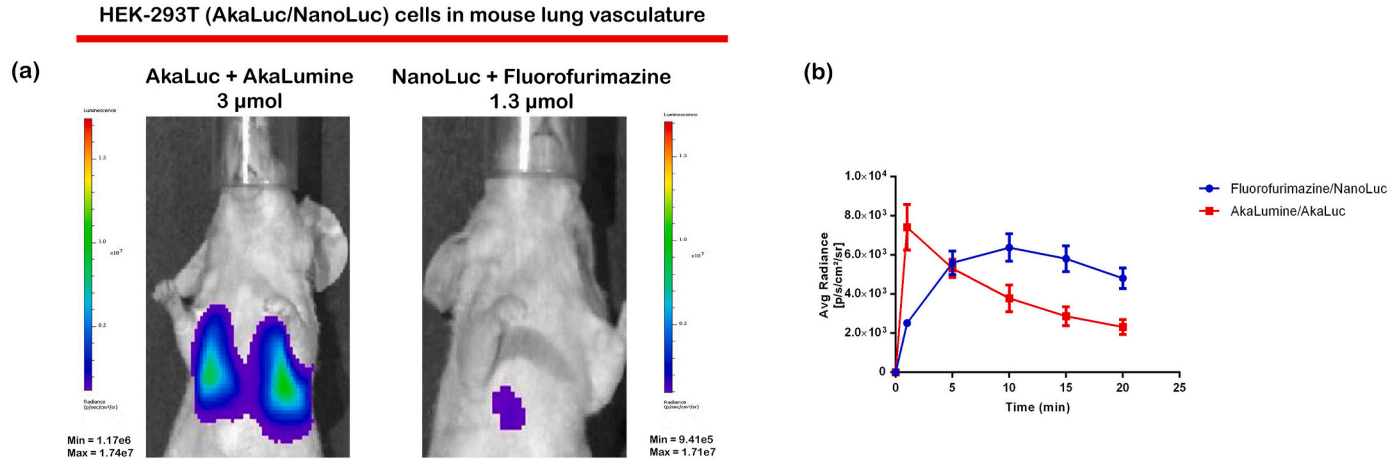

Fig. 6. Bioluminescent signal detection from a minuscule number of AkaLuc- and NanoLuc-expressing cells trapped in mice lung vasculature. Performance comparison of AkaLuc/AkaLumine *versus* NanoLuc/fluorofurimazine. (a) Bioluminescent imaging of mice intravenously injected with 10^5 HEK-293T cells expressing NanoLuc (right) with fluorofurimazine and (a) HEK-293T cells expressing AkaLuc (left) with AkaLumine. Substrates were administered 10 min post cell administration *via* an intraperitoneal injection; the animals were injected with 3 µmol AkaLumine and 1.3 µmol of fluorofurimazine. (b) Data was collected at the IVIS (1 s exposure time) and are presented as ±SD of $n = 3$ mice per substrate analogue.

BLI systems using cells expressing a known amount of luciferase injected subcutaneously in animals has been largely used in the past [22–25]. As for coelenterazine based substrates, it is generally known that they produce higher light output when injected intravenously [26]. Yet for repeated measurements, intraperitoneal *in vivo* injections should be preferred, given the reproducibility of the technique being less prone to misinjections.

A recent study reported that furimazine possibly displays toxic side effects, when administered repeatedly in small animals with doses exceeding 40 µg of substrate per day [27]. Furimazine, *inter alia*, is poorly soluble in aqueous solution, whereas D-luciferin salt can be dissolved to high concentrations in simple buffered saline [12,28], and repeatedly administered *via* intraperitoneal administration (i.p.). The preferred furimazine administration route is *via* intravenous (i.v.) injection, since higher photon emission is achieved. However, signals appear faster after substrate injection and show a rapidly decaying kinetic, limiting its use in certain areas of molecular imaging where a broader imaging window (longer substrate half-life) is required [13].

Based on our *in vivo* results we can clearly highlight on the importance of substrate solubility and bioavailability as the number one limitation for sensitive *in vivo* bioluminescent imaging [12]. The pairs, NanoLuc/fluorofurimazine (4.3 µmol and 333 nmol) and NanoLuc/hikarazine-003 (333 nmol), independent of the administration route, exhibited brighter (8 or 11-fold) *in vivo* BLI signals, than the standard NanoLuc/furimazine (333 nmol) pair. Moreover, it was clearly evident that the NanoLuc/fluorofurimazine (4.3 µmol and 333 nmol) pair was 11 or 3-fold brighter when administered intravenously or intraperitoneally, compared to the NLuc/hikarazine-003 pair (333 nmol). The photon generation from FFz is more prolonged and more intense, allowing *in vivo* tracking with the NLuc reporter over long time periods.

We also address *in vivo* coelenterazine analogues autoluminescence[17] by simply injecting the analogues in mice without the luciferase reporter being expressed. Such an approach provides accurate insight whether the compounds are prone to spontaneous light emission. Since dying cells expressing the NanoLuc reporter release the active luciferase in the bloodstream, which does not require any cofactors, and therefore can catalyze oxidation of its substrates and give background light. Our results demonstrate that NLuc substrate analogues (FFz, hikarazine-003) and furimazine itself also spontaneously oxidase, without the reporter being present in nude mice, and produce detectable light emission. This is important to take into account when performing imaging with furimazine and coelenterazine based analogues; performing a pre-scan and the proper positioning of animals is highly recommended in order to obtain accurate experimental results [12].

We next assessed the *in vivo* BLI performance of FFz with NLuc in deep tissue (lungs) and compared it to the near infra-red emission of

AkaLuc/AkaLumine pair, as a near-infrared BLI system reference with an emission peak at 650 nm.The luciferase expression was localised in mice lung vasculature by tail vein injection of NLuc and AkaLuc expressing cells [20,21]. AkaLuc/AkaLumine, yielded a 1.5-fold higher signal then FFz with NLuc. Nevertheless, here we demonstrate clearly that NLuc can be used for deep tissue imaging in small animals, when combined with the novel FFz.

Multiple BL reporters are derived from NLuc luciferase that uses furimazine, or its analogues as substrates, like Antares [6] Antares 2 and LumiScarlet [24,29]. These protein fusions exhibit emission wavelengths above 600 nm due to intramolecular bioluminescence resonance energy transfer (BRET), and therefore transmit light more easily through mammalian tissues. Moreover, using a different strategy, furimazine analogues can generate red-shifted emission when paired with NanoLuc [30,31], and therefore could enable more sensitive visualization in deep tissue. However, all of these bioluminescent systems do not show peak of emission in the near infrared region as the AkaLuc/AkaLumine system.

In conclusion, the combination of superior brightness and signal duration, given by FFz, enables bioluminescence imaging with NanoLuc luciferase, in superficial and deep tissue, making it applicable for studying diverse physiological events over a substantial period of time.

Author Contributions

L.M. and N.G. conceived and designed the whole study. N.G. performed all the experiments and analyzed the data. T.A.K and J.R.W. conceived designed and synthetized hydrofurimazine (HFz) and fluorofurimazine (FFz). K.M.P. performed the intravenous cell injections. G. Z. created the AkaLuc-HEK-293T stable cell line. N.G., T.A.K., J.R.W., C. L., and L.M. wrote the manuscript. L.M. supervised the project. All the authors read the manuscript and revised for important intellectual content.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2021.112128.

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