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NANOLuc/h-COELENTERAZINE: AN EFFECTIVE PAIR FOR DETECTING BIOLUMINESCENCE *IN VITRO* AND *IN VIVO*

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ABSTRACT

Introduction. Bioluminescent labeling of tumor cells is becoming a standard approach in preclinical studies of new anticancer drugs. Despite the wide variety of luciferases, many of them are not suitable for in vivo imaging.
Objective: to compare different NanoLuc substrates in vitro, and to demonstrate the feasibility of using the NLuc/h-coelenterazine pair for in vivo imaging of disseminated tumor cells with the IVIS Spectrum system.

Materials and Methods. The target cell line and T cells expressing chimeric antigen receptors (Chimeric antigen receptor, CAR) were generated via lentiviral transduction; luminescence was measured using a Luminoskan™ Microplate Luminometer; for in vivo imaging in an experimental mouse model of CAR T-cell therapy (NSG strain), the IVIS Spectrum in vivo imaging system was used.

Results. Different substrates were compared in in vitro assays by evaluating luminescence intensity and stability, and the use of h-coelenterazine paired with the Nluc luciferase was shown to enable in vivo imaging of cells in mice. A genetically modified cell line, Nalm6-NLuc-copGFP, was generated.

Conclusion. The results of comparing different substrates for the Nluc luciferase in in vitro and in vivo assays made it possible to identify an optimal enzyme/substrate pair that may serve as a valuable tool for studying the efficacy, safety, and toxicity of compounds and cell products being developed for anticancer therapy. The plasmid construct encoding Nluc can be used to modify other cell lines required for the development and characterization of new gene-therapy approaches.

Keywords: CAR T-cell therapy, bioluminescence, preclinical studies, furimazine, h-coelenterazine, native coelenterazine, NanoLuc luciferase

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NANOLuc/h-COELENTERAZINE: AN EFFICIENT PAIR FOR BIOLUMINESCENCE IMAGING *IN VITRO* AND *IN VIVO*

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ABSTRACT

Introduction. Bioluminescent labeling of tumor cells is increasingly becoming the standard approach in preclinical research on new anticancer drugs. However, despite the wide range of luciferases available, many are not well suited for in vivo imaging.

Aim: to compare different NanoLuc substrates in vitro and to demonstrate the practicality of the NLuc/h-coelenterazine pair for in vivo imaging of disseminated tumor cells using the IVIS Spectrum system.

Materials and methods. The target cell line and T cells expressing chimeric antigen receptors (CAR) were generated by lentiviral transduction. Luminescence was measured using a Luminoskan™ microplate luminometer, while in vivo imaging in an NSG mouse model of CAR T-cell therapy was carried out with the IVIS Spectrum system.

Results. Multiple substrates were compared in vitro for luminescence intensity and signal stability.

In addition, in vivo cell imaging in mice was demonstrated using h-coelenterazine in combination with Nluc luciferase. A genetically modified Nalm6-NLuc-CopGFP cell line was successfully generated.

Conclusion. Comparing multiple Nluc substrates in vitro and in vivo identified an optimal enzyme/substrate pair, which may serve as a useful tool for evaluating the efficacy, safety, and toxicity of compounds and cell-based products being developed for antitumor therapy. The plasmid encoding Nluc can also be used to engineer other cell lines needed to develop and characterize new gene-therapy approaches.

Keywords: CAR T therapy, bioluminescence imaging, preclinical study, furimazine, h-coelenterazine, native coelenterazine, NanoLuc luciferase **Acknowledgments.** The authors thank Andrey A. Gorchakov and Sergey V. Guselnikov, staff members of the Laboratory of Immunogenetics at the Institute of Molecular and Cellular Biology of the Siberian Branch of the Russian Academy of Sciences, for their help in preparing this publication. **Conflict of Interest.** The authors declare no conflicts of interest.

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Introduction

Conducting preclinical studies of antitumor therapy activity requires the ability to noninvasively track changes in tumor burden in the same animal over time. In the contex-t of developing cell-based immunotherapy, it is also desira-ble to have reliable tools to detect administe-red therapeutic cell products, and therefore, to enable simultaneous imaging of both tu-mor cells and the infused effector cells, because information limited to tumor-burden dy-namics alone is not sufficient for a proper and comprehensive in-terpretation of response to the therapy being administered [1–3].

The introduction into clinical practice of therapy, base-d on adoptive transfer of T-cells, expressing

Chimeric antigen receptors (Chimeric antigen receptor, CAR), used to treat patients with a range of B-cell–driven diseases, have revolutionized the field of immuno-oncology [4, 5]. Considerable expectations are also placed on adapting this approach for the treatment of solid tumors and autoimmune diseases [6–8]. Accordingly, rigorous preclinical evaluation of the activity and safety of CAR-cell products is a key requirement for success in this rapidly advancing area of molecular and cell-based medicine and is intended to help minimize health risks for patients taking part in such trials.

The simplest way to assess *in vivo* activity of CAR T-cell therapy is to genetically modify the cells of the engrafted-tumor in advance so they can produce fluorescence or luminescence. In practice, however, detecting fluorescent proteins in living laboratory animals is quite challenging [9] and the standard for these studies is bioluminescence detection. In that case, tumor cells are genetical-ly engineered to stably express a given luciferase, implanted into mice, and then luminescence intensity is measured regularly, starting immediately after administering the appropriate substrate.

Researchers have a broad toolkit of recombinant luciferase variants, most of which were derived from marine organisms [10, 11]. The substrates for these enzymes include coelenterazine [12], vargulin [11] or their analogs. Coelenterazine is commonly used with the luciferases Rluc (*Renilla reniformis*) and Gluc (*Gaussia princeps*) [13]; when it is oxidized, light is emitted with a peak in the blue region (450–485 nm) of the visible spectrum. For the luciferase Fluc (*Photinus pyralis*), widely used in studies that require bioluminescence detection, the substrate is D-luciferin, and the emission peak falls at 565 nm in the visible spectrum [14, 15]. Other widely used recombinant luciferases for different applications include Mluc [16], derived from *Metridia longa*, Cluc [17], derived from *Cypridina noctiluca*, as well as Vluc [18] and dDL [19]. However, the luminescence intensity of these reporters is often not sufficient, especially when detecting small numbers of engrafted cells.

In 2012, a unique enzyme–substrate pair (NanoLuc (=NLuc)/furimazine) was developed based on a mutated version of the catalytic subunit of a luciferase, originally identified in the deep-sea shrimp *Oplophorus gracilirostris* [20]. This luciferase has a specific activity that is 150 times higher than that of commonly used luciferases Fluc or Rluc [21]. In addition, NLuc offers outstanding chemical stability and thermal robustness, remaining active at temperatures up to 55 °C or after 15 hours in culture medium at +37 °C. It is also convenient that NLuc is ATP-independent and among the smallest luciferases by size (19 kDa). The range of NLuc use in modern biomedical and diagnostic applications is extremely broad, and this luciferase is a preferred reporter in experiments that require high signal brightness, stability, and low background luminescence.

Despite all the advantages of NLuc, reports also describe certain practical challenges when working with cells that express it, due to the low solubility and bioavailability of its substrate, furimazine [22, 23]. These issues arise, among other reasons, because the substrate dose cannot be increased: the maximum dose for small animals is 1.3 μmol in a polyethylene glycol–based buffer [24] and 0.016 μmol in phosphate-buffered saline [22], which limits the method's sensitivity.

Although modified furimazine variants have recently performed quite successfully in comparative studies, demonstrating that sensitivity can be improved when using them [23], commercial sources for these substrates remain limited. In this study, as an alternative that is also commercially available, we proposed using h-coelenterazine and comparing two coelenterazine forms— native coelenterazine and h-co-elenterazine— against furimazine in an *in vitro* assay, and to evaluate whether the NLuc/ h-coelenterazine pair can be used for in vivo imaging of disseminated tumor cells using the IVIS Spectrum system (Perkin-Elmer, USA).

Aim of this study was to compare different NanoLuc *in vitro* substrates, and to evaluate the feasibility of using the NLuc/h-coelenterazine pair for in vivo imaging of disseminated tumor cells using the IVIS Spectrum system.

Materials and Methods

Generation of a B-cell line Nalm6 with intracellular expression of NLuc

A dual-promoter CD-511 pCDH series vector (System Biosciences, USA), with the CMV–MCSEF1a-copGFP configuration, was selected as the cloning vector. The target construct, pCDH-nluc, was generated by cloning a codon-optimized sequence encoding the NLuc luciferase (pNL1.1 [Nluc] (Promega, USA)) using the unique XbaI and BamHI restriction sites (Thermo Fisher Scientifi c,

USA). Pseudotyped lentiviral particles were produced by cotransfecting HEK293T packaging cells with a three-plasmid mix: pCDH-nluc and the helper plasmids psPAX2 and pMD2.G, using calcium-phosphate transfection [25]. The supernatant, containing pseudoviral particles, was collected after 36 hours and the particles were pelleted by centrifugation for 1.5 hours at 35,000 g. The lentiviral pellet was resuspended in medium, flash-frozen, and stored in liquid-nitrogen vapor for no longer than 3 months.

The human B-cell acute lymphoblastic leukemia cell line Nalm6 (ATCC, USA), which naturally expresses CD19 on its surface, was cultured in IMDM (Thermo Fisher Scientifi c, USA) with 10% fetal bovine serum (Capricorn, Germany) at 37 °C in the presence of 5% CO_2. Nalm6 cells were transduced with pseudotyped lentiviral particles by spinoculation for 40 minutes at 32 °C at 600 g and at a ratio of 2 lentiviral particles per cell.

Several days later, once the fluorescent copGFP signal appeared in the population of transduced cells, single copGFP-positive cells were sorted on a «SH800» cell sorter (Sony, Japan) using Single cell (3 drops) mode. After expanding the sorted monoclonal populations for 2 weeks, the resulting monoclonal sublines were verified to be free of non-transduced (copGFP-negative) cells using a «BD CantoII» flow cytometer (Becton Dickinson, USA).

Measuring cell luminescence intensity in vitro

To determine luminescence intensity, cells from the Nalm6-Nluc-copGFP line generated as described were washed twice to remove culture medium with sterile Dulbecco's phosphate-buffered saline (DPBS, «PanEco», Russia), centrifuging for 4 min at room temperature at 400 g. Cells were then counted using an automated cell counter «LunaII» (LogosBio, South Korea) and seeded into wells of a 96-well plate (SPL, South Korea) ranging from 10 to 7290 cells/50 µL, using a threefold increase in cell number in adjacent wells. Cells were lysed by adding an equal volume of lysis buffer (DPBS, Triton-X up to 0.1%) for 10 min on an orbital shaker at 700 rpm. Each measurement point was run in triplicate. Substrates were diluted in phosphate-buffered saline to a final concentration of 30 µM immediately before starting the measurement. Substrate dispensing at 50 µL/well was performed automatically. Thus, after mixing with the lysed cells, the final substrate concentration was 10 µM. Signal detection was started immediately after substrate addition, recording for 0.5 sec and repeating this 50 times using the Luminoskan™ Microplate Luminometer (Thermo Fisher Scientifi c, USA).

Generation of CAR T cells

To generate CAR T-cell products, mono-nuclear cells were isolated from peripheral blood of a healthy donor by centrifugation on a Ficoll gradient ("PanEco", Russia), followed by selection and activation of T cells using magnetic be-ads CD3/CD28 Dynabeads (Thermo Fisher Scientifi c, USA). Isolated T cells were cultured in TexMACS me-dium TexMACS (Miltenyi Biotec, Germany) supplemented with 50 units/mL interleukin-2 and 7 ng/mL inter-leukin-15 (Miltenyi Biotec, Germany). Transduction with pseudoviral lentiviral particles, en-coding second-generation CARs targeting CD19 and PSMA (see below), was performed 36 ho-urs after isolation by spinoculation in the presence of 10 ng/mL protamine sulfate ("Sintez", Russia) for 40 minutes at 32 °C at 600 g. Transduction efficiency was assessed two days later by flow cytometry, staining with bio-tinylated protein L ("Biospecifica", Russia) and APC-streptavidin (Biolegend, USA).

In vivo imaging of tumor cells in an experimental mouse model of CAR T-cell therapy

For *in vivo* imaging, 6–8-week-old male NSG mice (n = 14) received tail-vein injections of 3×10^5 Nalm6-Nluc-copGFP cells/animal. Engraftment was monitored starting on day 5 after injection, and tumor burden was then assessed every 7 days. On day 6, animals were split into two groups and given 1×10^6 CAR T-cell products/animal; the experimental group received CD19-specific FMC63-CAR T cells, matching the tisagenlecleucel product (Novartis, USA) [26], while controls received J591-CAR therapy (a nonre-levant CAR specific for human PSMA) [27]. To visualize tumor cells, 20 µg h-coelenterazine (Nanolight Technologies, USA) in 100 µL phosphate-buffered saline was administered intraperito-neally, and 5 minutes later the animals were anestheti-zed with isoflurane. Luminescence was recorded for 180 sec., using the IVIS Spectrum in vivo imaging system (Perkin-Elmer, USA). Captured images were analyzed using Living Image software (Perkin-Elmer, USA). If animals developed pronounced pain syndromes, behavioral changes, or a high tumor burden

(based on imaging) the animal was removed from the experiment by decapitation. After removal of the animal from the experiment, bone marrow samples (femur) and blood were analyzed using flow cytometry to confirm the presence or absence of tumor cells in the samples. All animal procedures were approved by the local ethics committee of IMCB SB RAS, Decision No.002 dated 09.09.2024.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 6. Multiple comparisons were conducted using 2-way ANOVA; differences were considered significant at $P < 0.05$.

Results

Generation of Nalm6-Nluc-copGFP Cells

To generate the target cell line, we used the plasmid construct pCDH-nluc, which encodes NLuc luciferase under the control of a strong human cytomegalovirus promoter, and the green fluorescent protein copGFP from the *Pontellina plumata* copepod, driven by the strong constitutive promoter of the human EF1a gene (Fig. 1A). Nalm6 cells were transduced at an MOI of 2 using pCDH-nluc and packaging plasmids, which enable pseudotyping of lentiviral particles with the G protein of vesicular stomatitis virus.

Next, several monoclonal sublines were derived from the polyclonal population using-

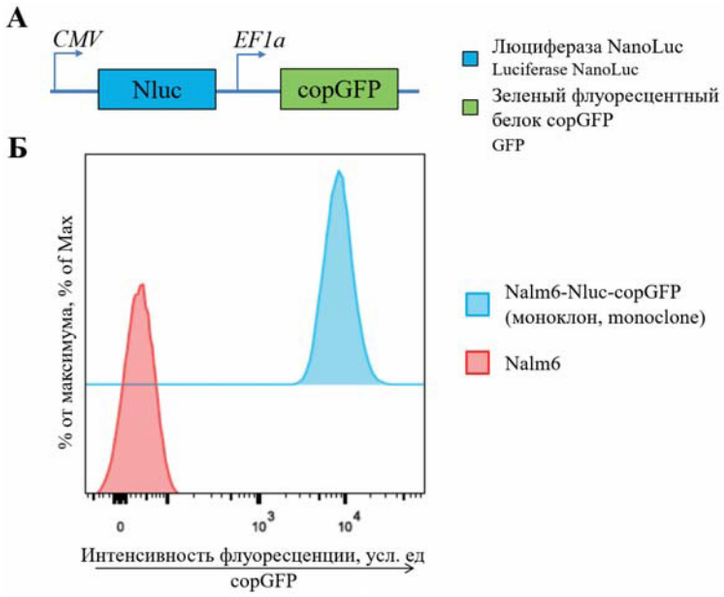

Figure 1. Generation and characterization of the target cell line. A — schematic showing the cassette structure encoding NLuc luciferase and the green fluorescent protein copGFP; B — the resulting monoclonal Nalm6-Nluc-copGFP line expresses copGFP (assessed by flow cytometry)

Figure 1. Development and characterization of the cell line. A — schematic representa-tion of the structure of the cassette encoding NLuc luciferase and the green fluorescent protein copGFP; Б — the resulting monoclonal line Nalm6-Nluc-copGFP expresses cop-GFP (analyzed by flow cytometry)

the «Sony SH800» cell sorter in single-cell sorting mode. The monoclonal lines were characterized by flow cytometry by assessing copGFP expression intensity, and one was selected—showing uniformly high fluorescence intensity (Fig. 1B). This clone was used for all subsequent experiments.

Functional In Vitro Assessment of Nalm6-NLuc-copGFP Cell Luminescence

To compare luminescent signal intensity as a function of substrate type and cell number, we seeded different numbers of cells (from 10 to 7290/well) at a fixed substrate concentration —10 μM. The results (Fig. 2A) showed a significantly stronger signal with h-coelenterazine than with furimazine. Luminescence with h-coelenterazine was approximately twofold higher than with furimazine. When native coelenterazine was used as the substrate, signal intensity was an order of magnitude lower, so we excluded it from further testing in subsequent experiments.

In addition to examining how luminescence intensity varied with cell number and establishing the sensitivity threshold of this method *in vitro*, we also evaluated the time-course decay of the luminescence signal over time, holding substrate concentrations at 10 μM and cell density at 2430 cells/well. The results of this test (Fig. 2B) showed that, unlike furimazine, which has high emission stability, using h-coelenterazine produces a sharp spike, followed by a gradual decline in luminescence intensity. Still, its brighter signal with subsequent stabilization, along with greater solubility and lower toxicity, make it a promising candidate for use in tests *in vivo*.

In vivo imaging of xenotransplanted Nalm6-Nluc-copGFP cells during treatment of mice with CAR T-cell products

To test whether xenotransplanted Nalm6-Nluc-copGFP cells could be visualized over time, we ran a pilot experiment, outlined in Figure 3A. After intravenous injection of 3×10^5 Nalm6-Nluc-copGFP cells into 14 male NSG immunodeficient mice, imaging and randomization into two groups were performed on day 5. The next day, one group received therapeutic CAR T cells that recognize CD19 on the surface of tumor

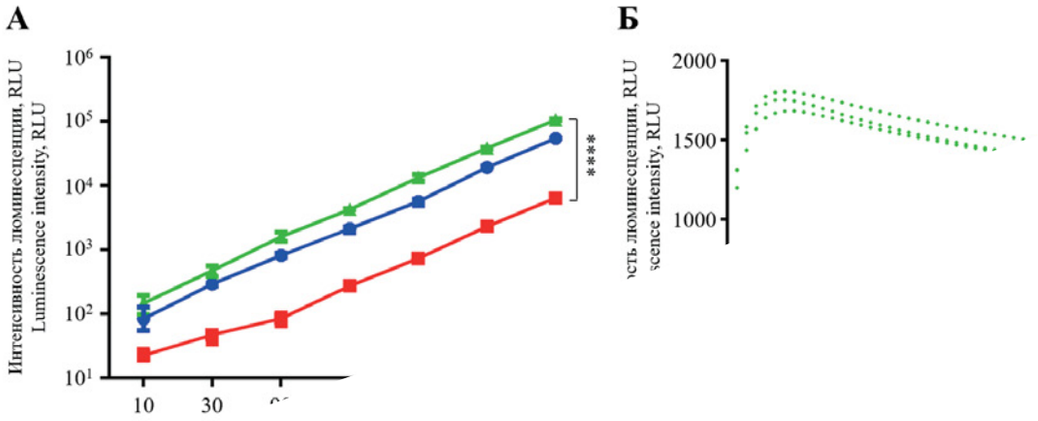

Figure 2. Comparison of different substrates in in vitro assays. A — comparison of integrated luminescence intensities over 25 seconds using different substrates (10 μM) as a function of the number of Nalm6-Nluc-copGFP cells. Statistical analysis of three replicates was performed using multiple comparisons with 2-way ANOVA (GraphPad Prism, v6, $p < 0.001$); B — mean bioluminescence intensity over time for each substrate at a concentration of 10 μM and 2430 Nalm6-NLuc-copGFP cells/well

Figure 2. In vitro comparison of different substrates. A — comparison of integrated luminescence intensities over 25 seconds using different substrates (10 μM) depending on the num-ber of Nalm6-Nluc-copGFP cells. Statistical analysis of three replicates was performed by multiple comparisons using 2-way ANOVA (GraphPad Prism, v6, $p < 0.001$); B — average bioluminescence intensity over time for each substrate at a concentration of 10 μM and 2430 Nalm6-NLuc-copGFP cells/well

cells (FMC63-CAR group), while others received control CAR T cells from the same donor, generated using the same method and activated/cultured *ex vivo* in the same way, with specificity for the PSMA protein, which is absent from the surface of tumor cells. Imaging was then performed weekly, on days 12, 19, 26, 33, 40, and 47 (Fig. 3B). Further disease progression was observed in control mice, whereas mice that received FMC63-CAR T cells showed stabilization of signal intensity, indicating that tumor growth had been brought under control.

Summary data on the mean luminescence intensity for both groups is shown in Figure 4. This experiment allowed us to answer yes to the question of whether the CAR T cells we generated have antitumor activity *in vivo* in a xenograft model, and also demonstrated that it is possible to image live animals and track the dynamics of NLuc-expressing tumor cells using an accessible and nontoxic substrate, h-coelenterazine.

Validating in vivo imaging results using flow cytometry

Because Nalm6-Nluc-copGFP cells express not only NLuc but also the fluorescent marker copGFP, their presence can be detected independently, and their phenotype can be characterized as needed by flow cytometry. Representative sample analysis data for one animal from the J591-CAR control group are shown in Figure 5. The results obtained indicated that Nalm6-NLuc-copGFP cells were detected in the bone marrow and peripheral blood of mice in full agreement with the in vivo imaging data.

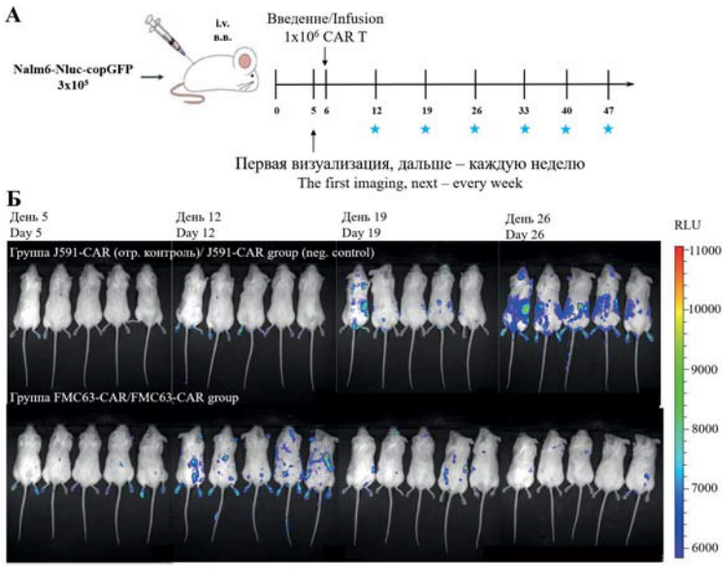

Figure 3. Live imaging of xenografted Nalm6-Nluc-copGFP cells during treatment of mice with CAR T-cell products. A — schematic overview of the experiment; B — in vivo imaging after intraperitoneal administration of 0.491 μmol (20 μg) h-coelenterazine in 100 μl phosphate-buffered saline in the control group of mice. Spectral data were collected for 3 min, 5 min after substrate administration. Representative images are shown for 5 of 7 mice in each group from day 5 through day 26

Figure 3. Live imaging of xenografted Nalm6-Nluc-copGFP cells during CAR T cell therapy in mice. A — schematic representation of the experiment; B — in vivo imaging after intraperitoneal administration of 0.491 μmol (20 μg) h-coelenterazine in 100 μl phosphate-buffered saline to the control group of mice. Spectral data were acquired for 3 min, 5 min after substrate administration. Representative images are shown for 5 of 7 mice in each group from days 5 to 26

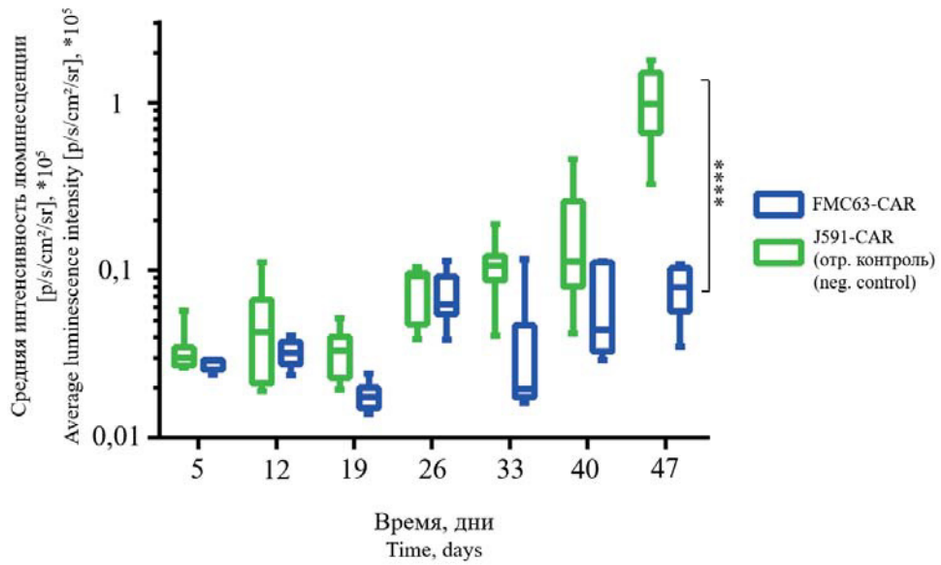

Figure 4. Changes in relative luminescence intensity over time after administration in each animal group (n = 7). On day 47, fluorescence intensity differs significantly; statistical analysis was performed using multiple comparisons with 2-way ANOVA (GraphPad Prism, v6, P < 0.001) **Figure 4.** Changes of relative luminescence intensity over time after administration, in each group of animals (n = 7). On day 47, the fluorescence intensity differs significantly, statistical analysis was performed using multiple comparisons with 2-way ANOVA (GraphPad Prism, v6, P < 0.001)

Discussion

One of the reasons, that prompted us to carry out this study, was that, according to the published literature, furimazine — a substrate specifically developed for the NLuc luciferase — may be toxic when administered repeatedly to small animals, and, in addition, it has poor water solubility, so the optimal route of administration for it remains intravenous injection [28]. With this route of administration, the signal rises more quickly and also fades faster, which further limits the use of furimazine for longer imaging processes [23]. In addition, it is well known, that for repeated measurements it is preferable to use intraperitoneal injections, given the method's greater reproducibility and lower susceptibility to errors.

Another potential substrate for NLuc — native coelenterazine — has been widely used in various experiments *in vivo*, because it is more bioavailable when administered intravenously [29], but in aqueous solutions it is not stable enough and degrades quickly. Unlike the native form, various C2-modified coelenterazine variants show greater stability in aqueous solutions [30]; in addition, for h-modification it has been shown to be preferable to use it with the BFP-aq protein [31]. There are no reports of using h-coelenterazine as a substrate for NLuc for in-vivo imaging applications.

In this study, we performed *in vitro* a comparison of three alternative substrates for the NLuc luciferase—native coelenterazine, h-coelenterazine

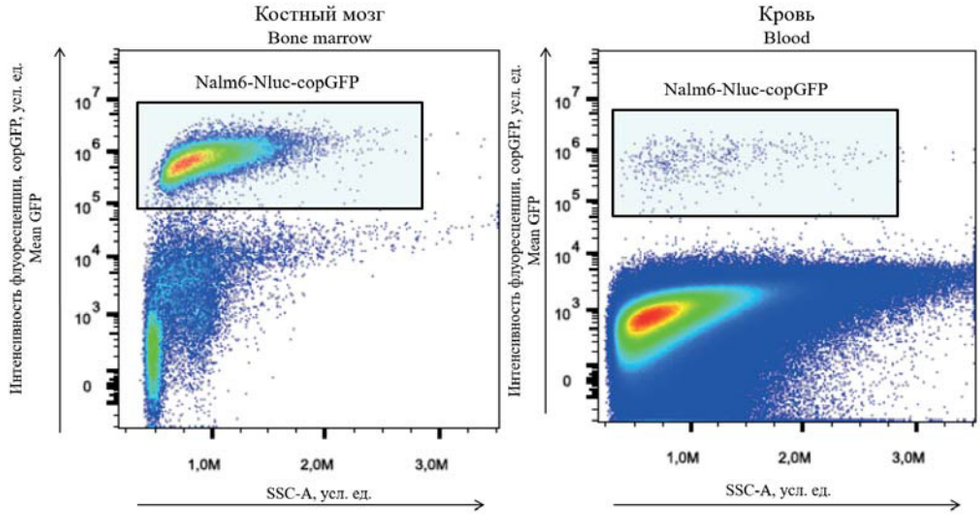

Figure 5. Flow cytometry analysis of bone marrow and blood samples from an animal in the control group (J591-CAR) **Figure 5.** Flow cytometry analysis of bone marrow and blood samples from a negative control animal (J591-CAR)

and furimazine. In experiments, h-coelenterazine showed significantly higher luminescence *in vitro* at an equimolar amount of substrate.

In addition to overall signal intensity, an important parameter in luminescence detection is its stability over time. Furimazine and its analogs produce an exceptionally stable signal for up to 30 min. after measurements begin [23]. Based on the data obtained, in the context of using *in vitro* together with NLuc, h-coelenterazine is a less preferred substrate than furimazine, due to greater signal dynamics in the first 5–7 sec. after addition. However, the signal from the NLuc/h-coelenterazine pair later stabilizes, which makes h-coelenterazine attractive for *in vivo* imaging, where detection starts much later, typically 3–5 min. after administration.

The wavelength of NLuc emission is almost 100 nm shorter than the emission wavelength of the widely used luciferase Fluc. In principle, this could lead to stronger tissue absorption of the NLuc luminescent signal, which in theory could obscure luminescence from deep-seated organs. In this study, no substantial distortion of luminescence patterns was observed compared with published data for Fluc luciferase [15]. Possibly **It is also possible that greater signal absorption is offset in the case of NLuc by its substantially higher emission intensity. Existing studies comparing imaging performance using both NLuc and FLuc within a single experiment in the context of xenotransplantation of U87 cells with concurrent expression of these two enzymes from a bicistronic construct also reported higher luminescence intensity for NLuc. That same experiment also demonstrated furimazine toxicity to cells and the need to identify an alternative substrate for in vivo**

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