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Comparative evaluation of coelenterazine analogs for in vivo bioluminescence imaging in preclinical oncology

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Conventional substrates often limit *in vivo* bioluminescence imaging due to suboptimal brightness, constrained dynamic range, poor solubility, and inconsistent emission kinetics, especially in deep or metastatic tumor models. In contrast to conventional ATP-dependent substrates, which are not ideal for preclinical oncology imaging, Hikarazine™ substrates from Synthelis Biotech are ATP-independent and have been engineered to deliver enhanced photon output, improved signal-to-background ratio, with optimized solubility ensuring excellent *in vivo* bioavailability for *Oplophorus gracilirostris*-derived luciferases. This makes Hikarazine™ particularly well-suited for longitudinal monitoring of tumor growth, treatment response, and functional phenotyping in small animals. In this application note, we present *in vivo* data comparing Hikarazine™ Z103 to Hikarazine™ Z108 and to another coelenterazine analog, Fluorofurimazine from Promega, in allograft mice via intraperitoneal injections at 0.75, 1.5, and 3 mg/kg. Key metrics including Biolum Index, 20-minute emission kinetics, and radiance-tumor volume correlations demonstrate Hikarazine™ Z103's superior brightness across all doses, stable kinetics post-administration, and strong linear correlations. Together, these results position Hikarazine™ Z103 as a powerful substrate of choice for sensitive, quantitative, and reproducible bioluminescence imaging in preclinical oncology.

INTRODUCTION

Bioluminescence imaging (BLI) represents a cornerstone of preclinical oncology for visualizing and quantifying tumor development in small animal models. This non-invasive method relies on the enzymatic oxidation of a luciferin substrate by a luciferase expressed in target cells, generating a detectable light emission. For preclinical studies, the most commonly used luciferases are those derived from *Photinus pyralis*, such as Firefly luciferase, which is activated by the D-luciferin substrate. *In vivo* BLI enables real-time longitudinal monitoring of cellular proliferation, metastasis, or therapeutic response.
However, conventional substrates like D-luciferin exhibit notable limitations in *in vivo* imaging, such as reduced bioavailability due to poor solubility and unstable emission kinetics. These constraints complicate precise quantitative analysis, particularly for assessing tumor dynamics over extended periods. Consequently, coelenterazine analogs compatible with luciferases derived from *Oplophorus gracilirostris* have emerged to address these shortcomings, offering improved tissue permeability and more stable emission.
Within the CCITADEL project of CELPHEDIA consortium, comparative tests were conducted to evaluate Hikarazine™ Z103 (Z103), Hikarazine™ Z108 (Z108), and the Fluorofurimazine (FFz) at different doses. These experiments, performed in groups of mice with tumors of varying sizes, measured the Biolum Index and radiance-to-tumor volume correlations. This application note details these data, highlighting the importance of selecting the optimal substrate to achieve *in vivo* bioluminescence imaging.

MATERIAL AND METHODS

-Nano-Glo® Fluorofurimazine – Promega (#N4100)
-Hikarazine™ Z103 – Synthelis Biotech (#SYN90006-1-01)
-Hikarazine™ Z108 – Synthelis Biotech (#SYN90007-1-01)
-37% Hydrochloric acid – Carlo Erba (#403871)
-Ethanol absolute – VWR (#VWRS30451.557)
-Dimethyl Sulfoxide – Sigma Aldrich (#D2438-50ML)
-Tris Buffer – (100mM, pH 8, Invitrogen (15504020))
-Mice (female, 9 weeks, Balb TG, janvier-labs)
-Colorectal cancer cell line (CT26-luc-OVA) expressing a luciferase derived from *Oplophorus gracilirostris*
-*In vivo* imaging system IVIS Lumina III – Revvity (#CLS136334)

1 Tumor induction in mice

Mice were used in the framework of the CCITADEL European project. Colorectal cancer CT26-luc-OVA syngenic tumors were induced by subcutaneous injection of 500,000 cells per mice. Following caliper-based measurement of tumor size, animals were stratified into small, medium, and large categories and then randomized into experimental groups to ensure standardized comparisons.

1.1 *In vivo* bioluminescence imaging

In this application note, three different substrates were compared: Hikarazine™ Z103, Hikarazine™ Z108 and FFz. Substrates were administered via intraperitoneal (IP) injection at three different doses: 0.75 mg/kg, 1.5 mg/kg, and 3 mg/kg. Post-injection, animals were anesthetized with 4% Vetflurane for induction and maintained at 2.5% during imaging, conducted 5 minutes later.

1.2 Image acquisition

Bioluminescence imaging was performed using an IVIS Lumina III, acquiring kinetic series of 10 to 20 images (1 image/minute) over 20–25 minutes post-injection. Regions of interest (ROIs) were defined over tumors to quantify average radiance (p/s/cm²/sr). A Biolum Index was then calculated for each condition: (Avg Radiance positive - Avg Radiance negative) / (2 × SD negative), stratified by tumor volume.

1.3 Data analysis

Correlation between radiance and real tumor volume, measured by caliper, was assessed via r^2 coefficient. Performances were compared based on brightness (Biolum Index), kinetic stability, and signal correlation to tumor size. Non-exploitable data points were excluded.

RESULTS AND DISCUSSION

1 Identification of the most performant substrate

The comparative analysis of substrates Z103, Z108, and FFz tested at different doses was performed using the Biolum Index, calculated for each condition and each tumor volume. The results show that compound Z103 exhibits the highest signal to noise ratio, regardless of the concentration tested.

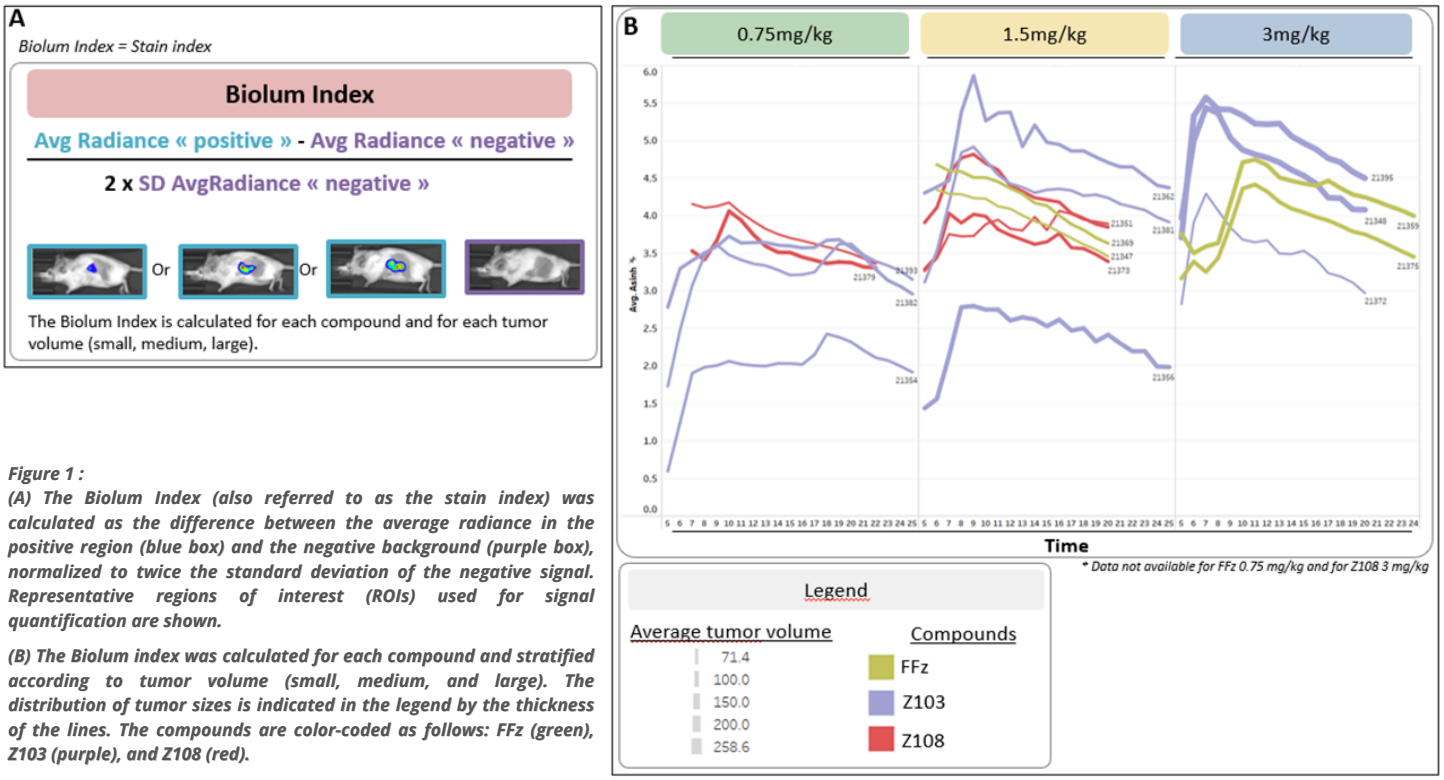

Figure 1 :
(A) The Biolum Index (also referred to as the stain index) was calculated as the difference between the average radiance in the positive region (blue box) and the negative background (purple box), normalized to twice the standard deviation of the negative signal. Representative regions of interest (ROIs) used for signal quantification are shown.

(B) The Biolum index was calculated for each compound and stratified according to tumor volume (small, medium, and large). The distribution of tumor sizes is indicated in the legend by the thickness of the lines. The compounds are color-coded as follows: FFz (green), Z103 (purple), and Z108 (red).

This superiority of compound Z103 could be explained by better bioavailability combined with more homogeneous diffusion in tumor tissues. The consistently higher Biolum Index across all concentrations also suggests excellent signal stability. The absence of usable data for certain conditions highlights the need to optimize experimental protocols or conduct these analyses with a larger number of animals.

2 Reliable quantification of tumor volume across a dose range

The ability to accurately estimate tumor size based on Z103 substrate dose was assessed by analyzing the correlation between measured radiance and actual tumor volume determined by caliper measurement. Strong correlations (high R^2 values) are observed at doses of 1.5 mg/kg and 3 mg/kg, while this correlation weakens at 0.75 mg/kg.

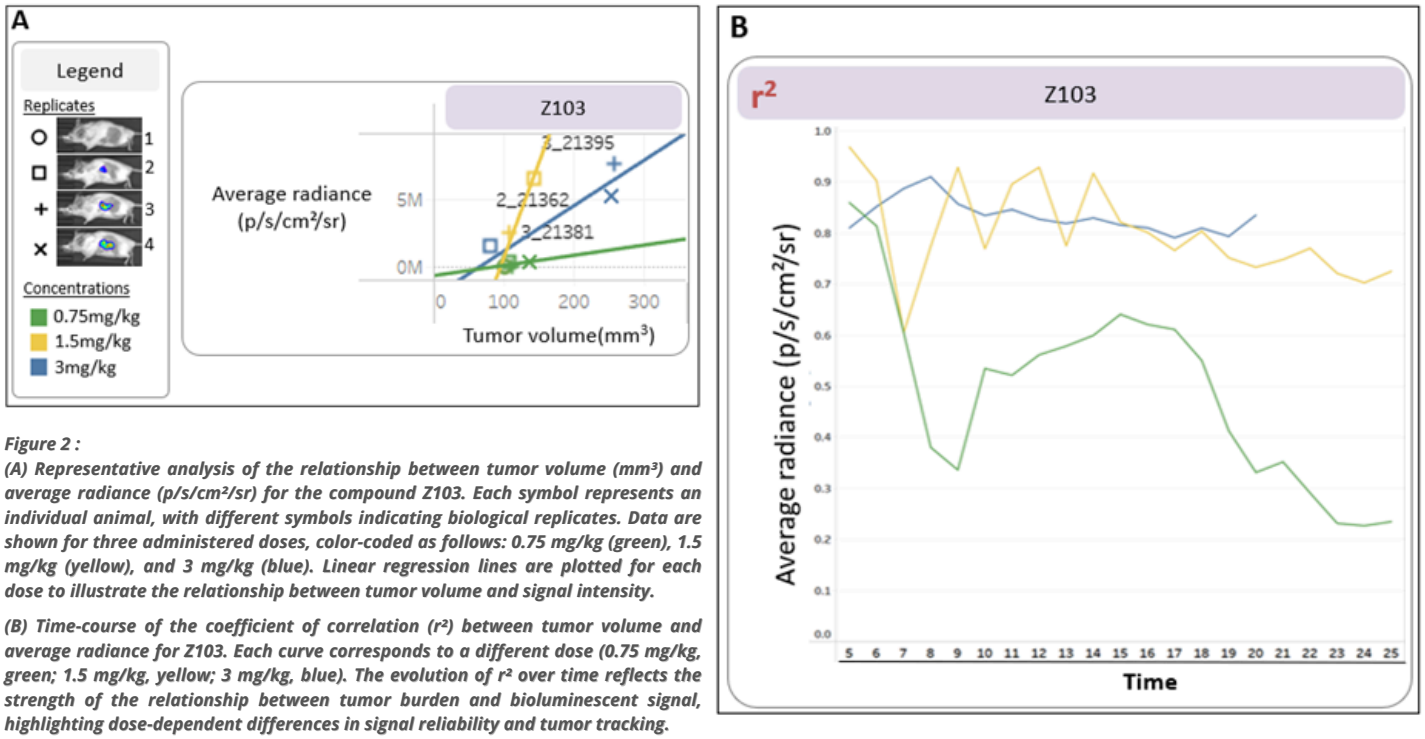

Figure 2 :
(A) Representative analysis of the relationship between tumor volume (mm³) and average radiance (p/s/cm²/sr) for the compound Z103. Each symbol represents an individual animal, with different symbols indicating biological replicates. Data are shown for three administered doses, color-coded as follows: 0.75 mg/kg (green), 1.5 mg/kg (yellow), and 3 mg/kg (blue). Linear regression lines are plotted for each dose to illustrate the relationship between tumor volume and signal intensity.

(B) Time-course of the coefficient of correlation (r²) between tumor volume and average radiance for Z103. Each curve corresponds to a different dose (0.75 mg/kg, green; 1.5 mg/kg, yellow; 3 mg/kg, blue). The evolution of r² over time reflects the strength of the relationship between tumor burden and bioluminescent signal, highlighting dose-dependent differences in signal reliability and tumor tracking.

These results suggest that Z103 dose can be reduced to as low as 1.5 mg/kg while maintaining reliable tumor burden quantification. This observation is particularly valuable for studies requiring repeated injections, helping to minimize potential effects from high doses. Conversely, the reduced correlation at the lower 0.75 mg/kg dose indicates insufficient signal sensitivity to accurately reflect tumor volume. An optimal balance between signal intensity and quantitative precision must therefore be determined based on the model studied.

CONCLUSIONS

In this study, Hikarazine™ Z103 proved to be the most performant bioluminescent substrate in an *in vivo* tumor model. This substrate exhibited superior luminescent signals across all injected doses and tumor sizes. The correlation between radiance and tumor volume was maintained at a reduced dose of 1.5 mg/kg, enabling precise tumor quantification even at early stages while minimizing costs and constraints associated with longitudinal studies.
Overall, these preliminary results position Hikarazine™ Z103 as the most optimal solution in currently commercially available compounds for sensitive, quantitative, and reproducible *in vivo* bioluminescence imaging in preclinical oncology.

References

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