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BRIEF ARTICLE

Multimodality Imaging of Cancer Superoxide Anion Using the Small Molecule Coelenterazine

Laura L. Bronsart,[1,2] Christian Stokes,[2] Christopher H. Contag[2,3]

[1]*Department of Biology, Stanford University, 318 Campus Drive, Stanford, CA, 94305, USA*
[2]*Department of Pediatrics, Stanford University, 318 Campus Drive, Stanford, CA, 94305, USA*
[3]*Departments of Radiology, Microbiology and Immunology, Stanford University, 318 Campus Drive, Stanford, CA, 94305, USA*

Abstract

Purpose: We evaluated the small molecule coelenterazine as a potential reporter of cancer-associated superoxide anion in cell culture and in mice.

Procedures: The superoxide anion concentrations of various cancer cell lines were quantified by coelenterazine chemiluminescence *in vitro*. Coelenteramide fluorescence was detected *via* flow cytometry and fluorescent microscopy. Coelenterazine was used for the *in vivo* detection of cancer-associated superoxide anion using the 4T1 breast adenocarcinoma mouse model.

Results: Various cell lines in culture demonstrated different superoxide anion concentrations, with a signal range of 3.15±0.06 to 11.80±0.24 times that of background. In addition to chemiluminescent detection of coelenterazine, we demonstrated fluorescent detection of coelenteramide within the cytoplasm of cells. 4T1 murine mammary adenocarcinoma tumors in mice demonstrated significantly higher 2.13±0.19-fold coelenterazine-based chemiluminescence than that of surrounding normal tissues.

Conclusions: Collectively, our results indicate that coelenterazine can be used to assay superoxide anion concentrations in cultured cancer cells and in tumors growing in mice.

Key words: Superoxide anion, Reactive oxygen species, Chemiluminescence, Bioluminescence, Fluorescence, Coelenterazine, Coelenteramide, Cancer

Introduction

Cancer cells have been shown to have higher levels of reactive oxygen species than do normal cells [1, 2]. The cause for these elevated levels is likely multifactorial and may be a cell intrinsic function associated with increased cellular metabolism and/or mitochondrial dysfunction, or a cancer cell extrinsic function due to local inflammation, reperfusion injury, or other sources of increased oxidase activity [3]. Reactive oxygen species are known to cause cellular damage when in excess, and in this regard, elevated levels are thought to predispose cells to malignant transformation [4–7]. However, reactive oxygen species, such as superoxide anion, are also recognized as cellular signaling molecules influencing a vast array of cellular events including cell proliferation, migration, and apoptosis [8–15]. Therefore, understanding the biological and pathophysiologic roles that reactive oxygen species play in tumor development, maintenance, and survival may offer insights into new treatment strategies that target the cancer-specific ROS requirements.

Novel, high-throughput tools for the detection and quantification of reactive oxygen species are necessary to further our understanding of their roles in oncogenesis and progression. Current methods are primarily limited to

Correspondence to: Christopher Contag; *e-mail:* ccontag@stanford.edu

fluorescent indicators designed for microscopic analysis of cultured cells or excised tissues [16–18]. Although such methods provide information at a cellular level, they can be inefficient and may not best represent cellular processes as they occur *in vivo*. The small molecule coelenterazine is known to react with the reactive oxygen species superoxide anion and peroxynitrite and produce a chemiluminescent signal [19–21]. Chemiluminescence imaging offers similar advantages to bioluminescence in regard to its imaging potential. Chemiluminescence, like bioluminescence, does not require excitation; thus, there is no background fluorescence or tissue penetrance concerns except in regard to emission. We have previously demonstrated that chemiluminescence from coelenterazine can be used to assay physiological superoxide anion production in cultured cells and in living animals (Bronsart et al, under review). In addition to being chemiluminescent, the by-product of the coelenterazine chemiluminescent reaction, coelenteramide, has been reported to be fluorescent [22, 23]. As such, we hypothesized that the small molecule coelenterazine may provide a method for multimodality imaging of cancer: a dynamic, chemiluminescent reporter of superoxide anion concentration in cultured cells and in living animals and a fluorescent reporter for the cellular and subcellular localization of the chemiluminescent reaction.

Materials and Methods

Materials

Native, *i.e.*, unmodified, coelenterazine was purchased from Nanolight (#303). Dihydroethidium was purchased from Life Technologies (#D11347).

Cell Culture

Cell lines were cultured in RPMI-1640 with 10 % fetal bovine serum (Invitrogen), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. INS-1 cell media was additionally supplemented with 50 μmol/l 2-mercaptoethanol.

In Vitro Coelenterazine Imaging

Cell lines were split 48 h prior to the experiment to achieve a final confluence of approximately 60 % on the day of the experiment. Cells were trypsinized, washed twice in phosphate buffered saline (PBS), and resuspended in Krebs buffer (140 mmol/l NaCl, 30 mmol/l Hepes, 4.6 mmol KCl, 1 mmol/l $MgSO_4$, 0.15 mmol/l Na_2HPO_4, 5 mmol/l $NaHCO_3$, and 2 mmol/l $CaCl_2$) supplemented with 4 mmol/l D-glucose. Cells were added to wells of a 96-well black plate (5×10^5 cells per well). All samples were prepared in triplicate. The total flux (p/s) was quantified for each well using Living Image software.

In Vitro Dihydroethidium Detection

Cell lines were split 48 h prior to the experiment to achieve a final confluence of approximately 60 % the day of the experiment. The cells were trypsinized, washed twice in PBS, and resuspended in Krebs buffer (140 mmol/l NaCl, 30 mmol/l Hepes, 4.6 mmol KCl, 1 mmol/l $MgSO_4$, 0.15 mmol/l Na_2HPO_4, 5 mmol/l $NaHCO_3$, and 2 mmol/l $CaCl_2$) supplemented with 4 mmol/l D-glucose. Cell numbers were adjusted to a final concentration of 5×10^6 cells/ml. Dihydroethidium staining was completed as previously described [24]. Briefly, dihydroethidium was suspended in dimethyl sulfoxide (DMSO) to a concentration of 10 mM. Dihydroethidium or equal volume of vehicle control was added to cell suspensions to a final concentration of 5 μM and incubated for 10 min protected from light. Cell suspensions were washed twice with PBS, counted, and resuspended to a concentration of 5×10^6 cells/ml and fluorescence detected using the Biotek® Synergy 4 plate reader using an excitation of 490 nm and an emission of 590 nm.

Fluorescence Microscopy

HeLa cells were collected by trypsinization, plated on glass-bottom plates at a density of 1×10^5 cells/ml media, and incubated for 24 h. Cells were then washed twice with PBS and incubated in Krebs buffer supplemented with 4 mM glucose. Coelenterazine or equal volume of vehicle control was added to a final concentration of 10 μmol/l and cells were incubated for 2 h in a total volume of 1 ml. Cells were then counterstained with NucBlue® Live ReadyProbes® (Life Technologies #R37606). Imaging was performed using the EVOS® Cell Imaging System. Coelenterazine fluorescence was detected using the green-fluorescent light cube (excitation: 470/22 and emission: 510/42).

Flow Cytometry

HeLa cells were collected following trypsinization, washed twice in PBS and resuspended in Krebs buffer supplemented with 4 mM glucose. Coelenterazine- and vehicle control-treated samples were incubated with 10 μmol/l of coelenterazine for 1 h at 37 °C. Samples were stained with the LIVE/DEAD® Fixable Far Red Cell Stain (Life Technologies #L10120). Cells were fixed and stored in 0.37 % NBF at 4 °C overnight. Flow cytometry was completed at the Stanford Shared FACS Facility.

Ethics Statement

All animal studies were completed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Stanford University.

4T1 Animal Model

Tumor Implantation

Murine mammary adenocarcinoma cells (4T1) were implanted into the subcutaneous right mammary fat pad in each of ten females,

BALB/c, age 6 weeks, using a total cell number of 1×10^6 or mice were given a vehicle-only control. Implantation occurred on day 0, relative to imaging.

Mouse Imaging

Mice were imaged on the day of 4T1 inoculation (day 0) and at 3 and 6 days post-implantation. Coelenterazine was resuspended in 100 % ethanol to a concentration of 5 mg/ml [25]. Individual aliquots of coelenterazine were stored on dry ice and protected from light. At the time of imaging, an aliquot was diluted to a final concentration of 1 mg/ml in a 5 % glucose solution. Each mouse was anesthetized with isoflurane inhalant anesthetic and intravenously administered 5 mg/kg native coelenterazine. Mice were immediately imaged using the IVIS Spectrum optical imaging platform. The average radiance (p/s/cm^2/sr) from the tumor implantation region was selected and quantified using the Living Image software.

Statistical Analysis

Data was presented as the mean±SEM. Significance was determined by the Student's two-tailed *T* test and defined as $P\leq0.05$. The Pearson's correlation method was used to determine the R^2 and the significance of linear correlations.

Results

Superoxide Anion Concentrations Vary Among Cancer Cell Lines

Addition of native coelenterazine (final concentration of 10 µmol/l) to a variety of cancer cell lines was used to image and quantify coelenterazine chemiluminescence using a CCD camera (IVIS). All cancer cell lines had significantly greater ($P\leq1.75\times10^{-19}$) chemiluminescence than the buffer control; however, there was substantial variability among the cell lines (Fig. 1a, b). The human cervical cancer cell line HeLa had significantly greater ($P\leq4.76\times10^{-18}$) signal than any other line tested. Both rat β cell lines RIN-5F and INS-1 also had significantly higher levels ($P\leq4.76\times10^{-18}$) of chemiluminescence followed by the mouse mammary adenocarcinoma cell line 4T1. Interestingly, cell lines of similar tissue origin had similar concentrations of superoxide anion. RIN-5F and INS-1 cells are both rat β cell lines and demonstrated similar chemiluminescent signal intensity. Additionally, the 293FT cell line had similar superoxide anion concentrations as the original HEK 293 cell line.

To determine if the detection of superoxide anion with coelenterazine was consistent with superoxide anion concentrations as determined by another methodology, cancer cell lines were treated with either coelenterazine or the commonly used superoxide anion detection agent dihydroethidium, and chemiluminescence or fluorescence was detected. Coelenterazine-mediated chemiluminescence significantly correlated with dihydroethidium-mediated fluorescence with an *R*-squared value of 0.8957 (P=0.0004) (Fig. 1c).

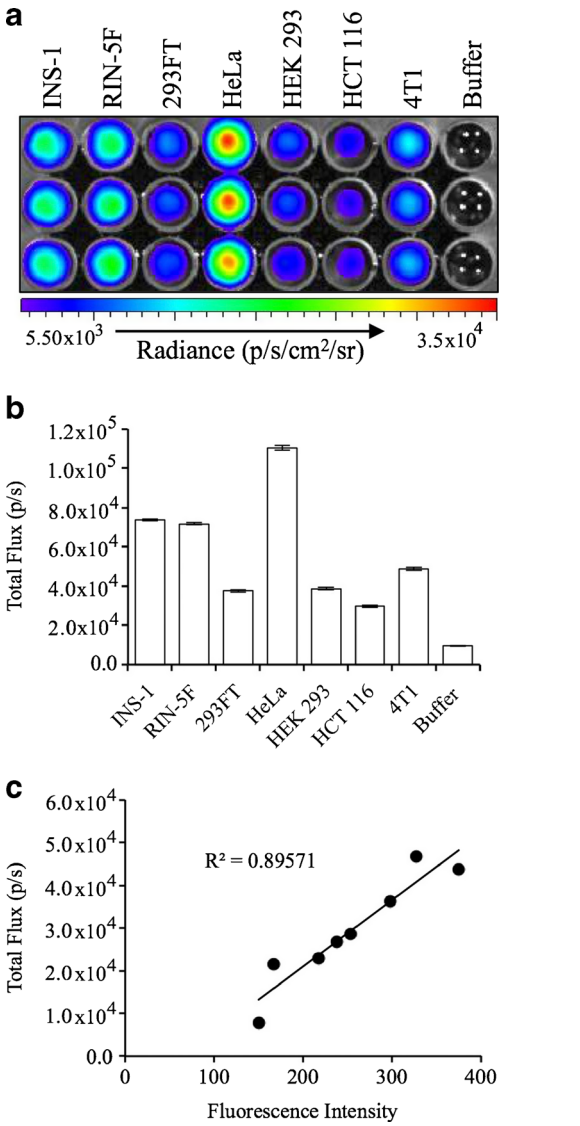

Fig. 1 The concentration of superoxide anion varies significantly among cancer cell lines. **a** 5×10^5 cells of each line were imaged with 10 µmol/l native coelenterazine. The 4T1 cell line is a mouse breast adenocarcinoma line, HCT 116 is a human colon carcinoma line, HeLa is a human cervical cancer line, HEK 293 and 293FT are human embryonic kidney lines, and RIN-5F and INS-1 are rat β cell lines. **b** Quantification of coelenterazine chemiluminescence for each cell line (n=3). (*Error bars* represent the mean±SEM.) **c** The chemiluminescence or fluorescence of 5×10^5 cells of each line in (**a**) was quantified following 60 min of incubation with 10 µmol/l native coelenterazine or 5 µmol/l dihydroethidium. The data was plotted as the coelenterazine-mediated chemiluminescence total flux (p/s) *versus* the dihydroethidium fluorescence intensity, and the *R*-squared value showed a good correlation with value of 0.8957.

Fluorescence Microscopy Can Be Used to Detect Coelenteramide

Previous studies have shown that the coelenterazine reaction by-product coelenteramide is fluorescent [22, 23]. We aimed to use this optical property to determine the cellular localization of coelenteramide. Flow cytometry offered a rigorous method to determine if the coelenteramide was intracellular as it provides single-cell analysis under significant flow conditions. HeLa cells were treated with coelenterazine or vehicle control. Cells treated with coelenterazine demonstrated detectable fluorescence over background (Fig. 2a). Since flow cytometry offered the capability of high-throughput, single-cell analysis, we next tested if the observed coelenteramide fluorescence was detected in populations of live or dead cells. The majority, 95 %, of cells were both alive and coelenteramide positive at the time of fixation (Fig. 2b). Furthermore, fluorescent microscopy of HeLa cells treated with 10 μmol/l native coelenterazine and counterstained with DAPI nuclear stain revealed coelenteramide fluorescence localized to the cell cytoplasm (Fig. 2c).

Coelenterazine Detects Cancer-Associated Superoxide Anion In Vivo

Despite reactive oxygen species playing a significant role in both health and disease, including cancer, little work has been done studying superoxide anion *in vivo* due to the limited number of tools available. Having assessed coelenterazine as a tool for the *in vitro* analysis of cancer cells, we next evaluated coelenterazine as an *in vivo* agent for the detection of cancer-related superoxide anion. The 4T1 murine mammary adenocarcinoma cell line was implanted into the right, inguinal mammary tissue of BALB/c mice. There was substantial upper abdominal and thoracic signal observed in all mice. This was presumed to

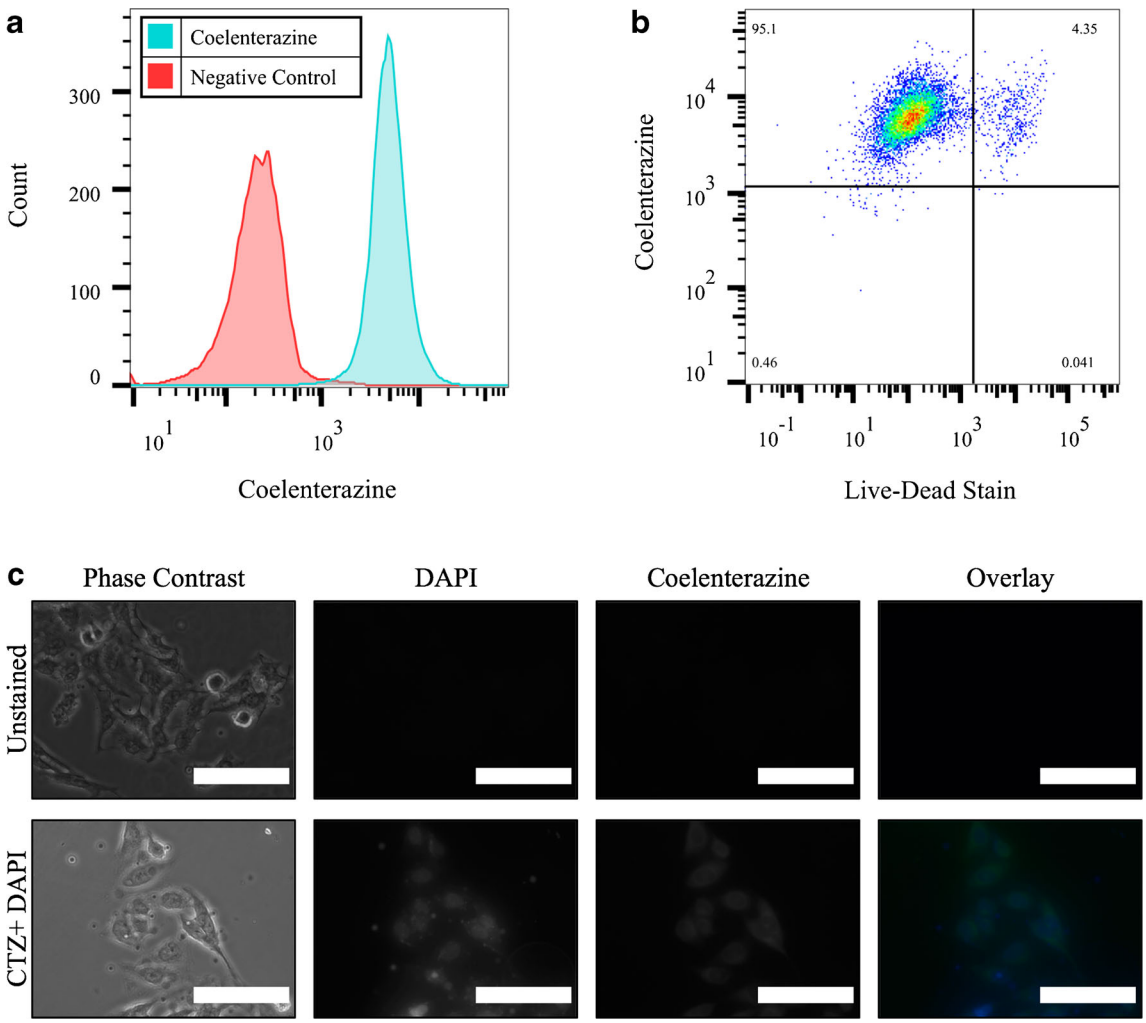

Fig. 2 Coelenteramide fluorescence is detectable by flow cytometry and fluorescent microscopy. **a** Flow cytometry analysis of unstained and coelenterazine-treated HeLa cells. **b** Flow cytometry of coelenterazine-treated and live-dead stained cells. c Fluorescence microscopy of unstained (*left*) and coelenterazine-treated and DAPI-stained (*right*) HeLa cells. *CTZ* indicates coelenterazine treated, and *scale bar* is 100 μm.

be pancreatic β cell and lung mediated as previously described by our group (Bronsart et al, under review). Despite this limitation, the 4T1 cells in mice showed discernable chemiluminescence from the implant region (Fig. 3a). The chemiluminescent signal intensity significantly increased from day 0 to day 3 (P=0.0005) and from day 0 to day 6 (P=0.0003) following implantation. Additionally, the signal was significantly elevated in the 4T1 group compared to the control group on days 3 (P=0.0010) and 6 (P=0.0001) (Fig. 3b). These results suggested that, depending upon the implant location, coelenterazine may be a useful tool for the *in vivo* detection of superoxide anion as it relates to tumor biology.

Discussion

The aim of this research was to determine if coelenterazine would enable studies of superoxide anion in cancer cells and chemiluminescent detection of cancer *in vivo* and if the reaction by-product, coelenteramide, would be detectable by fluorescent imaging. This was addressed by imaging the chemiluminescence and fluorescence of coelenterazine in cells in culture. Additionally, application of the 4T1 murine mammary adenocarcinoma model was used for *in vivo* imaging. Collectively, our results indicate that coelenterazine may be a useful tool for evaluating the role of superoxide anion in cancer cell biology and its role in pathology.

Various Cancer Cell Lines Have Different Concentrations of Superoxide Anion

We had previously demonstrated that coelenterazine could be used to dynamically detect superoxide anion concentrations in cells in culture and that a significant source of the superoxide anion was oxidative phosphorylation (Bronsart et al, under review). Interestingly, different concentrations of superoxide anion were detected from a variety of cancer cell lines. Additionally, although our study was limited in scope, there was evidence that cell lines of similar tissue origin may have similar superoxide anion concentrations. These results suggest that cellular superoxide anion concentrations may be determined, in part, by cellular heritage. Additionally, it emphasizes that each cancer is unique and must, therefore, be evaluated on an individual basis and that differences in cellular superoxide anion levels may be indicative of differences in biological function.

Coelenteramide Fluorescence Is Detectable by Flow Cytometry and Microscopically

Coelenteramide is the fluorescent by-product of the coelenterazine reaction [22, 23]. Despite the weak signal, its fluorescent nature enabled confirmation of the cellular location of the coelenterazine reaction. The flow cytometry results support that the reaction was occurring intracellularly and the microscopy indicates a cytoplasmic localization. This result is consistent with other fluorescent indicators of superoxide anion [26]. As such, coelenterazine may serve as a multimodal reporter of superoxide anion concentrations. The chemiluminescent signal serves as a more sensitive, dynamic readout of superoxide anion concentrations while the fluorescence serves as an indicator of cellular localization.

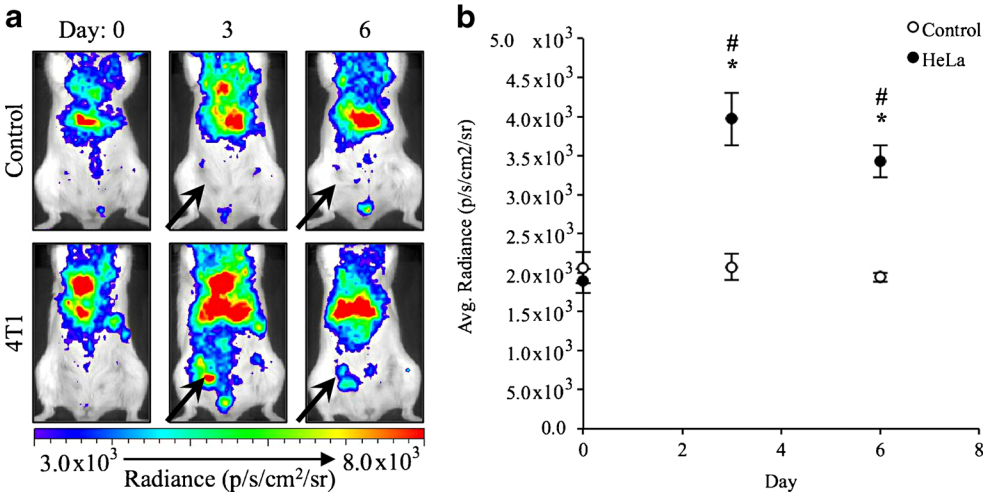

Fig. 3 Coelenterazine chemiluminescence is detectable from tumor sites. **a** Images of a representative control and 4T1-implanted mouse prior to (day 0) and 3 and 6 days post-implantation. *Arrows* indicate the site of vehicle control or 4T1 cancer cell implantation. **b** Quantification of the chemiluminescent signal intensity as the average radiance over a region of interest drawn over the tumor or vehicle control implantation sites (n=5). (*Error bars* represent the mean±SEM, *asterisk* denotes a significant difference between the groups of P<0.001, and *number sign* denotes a significant difference between HeLa-engrafted mice when compared to day 0 of P<0.0005.)

Coelenterazine Detects Increased Superoxide Anion Concentrations at Tumor Sites

Coelenterazine chemiluminescence was detectable *in vivo* at tumor sites 3 and 6 days post-implantation. Unfortunately, there was substantial background signal due to the previously described physiologic superoxide anion production (Bronsart et al, under review). As such, the *in vivo* use of coelenterazine for imaging superoxide anion production in orthotopic tumor models is limited. Additionally, this method would likely not be applicable to the study of metastatic lesions as most metastases occur in similar locations as the observed background chemiluminescence. Despite these limitations, coelenterazine chemiluminescence imaging enabled *in vivo* detection of extra- and intracellular cancer-related superoxide anion concentrations. An additional chemiluminescent reporter of superoxide anion exists, lucigenin; however, unlike coelenterazine, it is restricted to the extracellular space [27]. It is still unknown if coelenteramide fluorescence can be detected within excised tissues following *in vivo* administration. Such a result would enable *in vivo* chemiluminescent imaging followed by microscopic analysis of excised cells and tissues to determine the cellular localization of the *in vivo* chemiluminescence signal production.

In conclusion, coelenterazine offers a unique opportunity to dynamically assess the global production of superoxide anion as it relates to cancer development, progression, and survival both *in vitro* and *in vivo*. As well as being useful for the study of cancer biology and pathology, it may provide a reliable high throughput means to assess how therapies, chemotherapeutic, immunological, and radiological, affect superoxide anion concentration and how this relates to disease progression. Reporters for biological molecules are most useful when they can be applied in a biologically relevant context; coelenterazine provides that opportunity.

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Conflict of Interest. The authors declare that they have no competing interests.

Ethics Statement. All applicable institutional and/or national guidelines for the care and use of animals were followed.

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