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Ferroptosis induces nucleolar stress as revealed by live-cell imaging using thioflavin T

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ABSTRACT

Nucleolar stress induced by stressors like hypoxia, UV irradiation, and heat shock downregulates ribosomal RNA transcription, thereby impairing protein synthesis capacity and potentially contributing to cell senescence and various human diseases such as neurodegenerative disorders and cancer. Live-cell imaging of the nucleolus may be a feasible strategy for investigating nucleolar stress, but currently available nucleolar stains are limited for this application. In this study using mouse hippocampal HT22 cells, we demonstrate that thioflavin T (ThT), a benzothiazole dye that binds RNA with high affinity, is useful for nucleolar imaging in cells where RNAs predominate over protein aggregates. Nucleoli were stained with high intensity simply by adding ThT to the cell culture medium, making it suitable for use even in damaged cells. Further, ThT staining overlapped with specific nucleolar stains in both live and fixed cells, but did not overlap with markers for mitochondria, lysosomes, endoplasmic reticulum, and double-stranded DNA. Ferroptosis, an iron-dependent nonapoptotic cell death pathway characterized by lipid peroxide accumulation, reduced the number of ThT-positive puncta while endoplasmic reticulum stress did not. These findings suggest that ferroptosis is associated with oxidative damage to nucleolar RNA molecules and ensuing loss of nucleolar function.

1. Introduction

The nucleolus serves as the primary site for ribosomal RNA (rRNA) synthesis, covalent RNA modifications, and assembly of ribonucleoprotein complexes (Gerbi et al., 2003; Boisvert et al., 2007; Bogurcu--Seidel et al., 2023). In addition, the nucleolus is strongly implicated in cell cycle control and stress responses. Oxidative stress, such as hydrogen peroxide, is involved in the inactivation of the transcription factor TIF-IA, leading to the down-regulation of rRNA synthesis (Mayer et al., 2005). Cellular stressors such as UV irradiation, heat shock, hypoxia, and chemical toxicity can impair rRNA transcription, leading to a condition termed nucleolar stress, and recent evidence has implicated this condition in neurodegenerative disorders, viral infection, cancer, and premature aging (Parlato and Kreiner, 2013; Maehama et al., 2023). For instance, immunohistochemical staining of the nucleolar protein nucleophosmin revealed significant nucleolar disruption in dopamine neurons from patients with Parkinson's disease (Rieker et al., 2011). It has also been suggested that small nucleoli are a visible hallmark of cellular longevity and metabolic health, and that molecules associated with nucleolar function may serve as predictive biomarkers of life expectancy (Tiku et al., 2017). Therefore, an easy-to-use method for visualizing the nucleolus will facilitate exploration of these associations and potential therapeutic interventions for human diseases.

A variety of imaging techniques are used to reveal distinct aspects of nucleolar morphology and composition, including transmission electron microscopy, differential interference contrast imaging, and immunofluorescence labeling of nucleolar proteins (Boisvert et al., 2007). In addition, several fluorescent probes specifically designed for staining RNA, such as Nucleolus Bright Red (Dojindo), Nucleolus Bright Green (Dojindo), SYTO RNA select (ThermoFisher) for fixed samples, and

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PhenoVue 512 Nucleic acid stain (PerkinElmer) for live and fixed cells, can be used to visualize nucleoli. However, each has limitations, including high cost, spectral overlap with other common fluorophores, and poor cell membrane permeability.

The benzothiazole dye thioflavin T (ThT) is widely used for the identification and quantification of amyloid fibrils in vitro (Naiki et al., 1989). In addition, many unrelated DNAs and RNAs that contain G-quadruplex motifs bind ThT and strongly activate its fluorescence (Mohanty et al., 2013; Sugimoto et al., 2015). This characteristic prompted us to investigate the potential of ThT for nucleolar imaging in live cells. In this study, we demonstrate that ThT stains nucleoli with high efficiency and good selectivity in both healthy and damaged cells when added to culture medium containing fetal bovine serum (FBS). The mouse hippocampus-derived cell line HT22, a well-established cellular model for studying major intracellular stress response pathways including oxidative stress and endoplasmic reticulum stress (Lewerenz et al., 2018; Hasegawa et al., 2022), is employed to investigate the impact of oxidative stress, such as ferroptosis and hydrogen peroxide, on nucleolar staining.

2. Materials and methods

2.1. Materials

ThT was obtained from Tokyo Chemical Industry (Tokyo, Japan), Nucleolus Bright Red from Dojindo Laboratories (Kumamoto, Japan), erastin from MedChemExpress (Monmouth Junction, NJ, USA), and bisBenzimide H 33342 trihydrochloride (Hoechst 33342), hydrogen peroxide, RSL3, tunicamycin, and thapsigargin from Sigma-Aldrich (St. Louis, MO, USA). Nucleus Bright Red, erastin, RSL3, tunicamycin, and thapsigargin was dissolved in cell culture-grade dimethylsulfoxide.

2.2. Cell culture

The immortalized mouse hippocampal cell line HT22 (RRID: CVCL_0321), a generous gift from the late Dr. David Schubert (The Salk Institute, La Jolla, CA, USA), was cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemicals, Osaka, Japan, Cat# 041–29775) supplemented with 5% FBS (Nichirei Biosciences, Tokyo, Japan) at 37 °C in a 5% CO_2 incubator. Similarly, HEK-293 cells (ATCC, Manassas, Virginia, USA) were cultured in DMEM (Wako Pure Chemicals, Cat# 043–30085) supplemented with 8% FBS under the same conditions. Both cell lines were replaced either at passage 30–40 or every 3 months from the original frozen stocks.

2.3. Cell imaging

Cells were seeded at 2×10^5 per dish on 35-mm glass-bottom dishes (AGC Techno Glass, Shizuoka, Japan) and incubated in DMEM supplemented with FBS (5% or 8% as indicated) for 24 h. For live-cell imaging of the nucleolus, ThT (5 μM) was added directly to the medium for 30–60 min. For fixed cell staining, cells were first washed twice with phosphate-buffered saline (PBS) and fixed with 4% Paraformaldehyde Phosphate Buffer Solution (FUJIFILM Wako Pure Chemicals, Osaka, Japan) for 30 min at room temperature. Subsequently, fixed cells were washed twice with PBS, incubated with a permeabilizing solution (0.5% Triton X-100, 3 mM EDTA/PBS) for 30 min on ice under gentle shaking, rewashed twice with PBS, and finally incubated with the detection reagent (5 μM ThT, 5 μM Nucleolus Bright Red/PBS, or both) for 30 min in the dark. The subcellular distribution of fluorescence emission in both live and fixed cells was analyzed using a Keyence BZ-X810 microscope equipped with an optical sectioning algorithm.

2.4. Western blotting

HT22 cells were treated as indicated, and cell pellets were lysed in Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], and 10% glycerol) by sonication. The protein concentration in each lysate preparation was measured using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and γ-globulin as the standard. Equal amounts of protein (10 μg) were separated on SDS-polyacrylamide gels by electrophoresis and subsequently transferred to nitrocellulose membranes (Hybond-enhanced chemiluminescence, GE Healthcare UK, Buckinghamshire, England). The membranes were blocked with 5% nonfat dry milk in PBS-0.05% Tween-20 and then probed with the following primary antibodies overnight at 4 °C: anti-nucleophosmin (B23/NPM1) antibody (1:5000; Proteintech, Rosemont, IL, USA, RRID:AB_2155162), anti-glutathione peroxidase 4 (GPX4) antibody (1:1000; abcam, Cambridge, UK, RRID:AB_10973901), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000; OriGene Technologies, Rockville, MD, USA, cat.# ACR001P) as the gel loading control. The immunolabeled membranes were then probed with a secondary antibody for 90 min at room temperature (RT) and target bands were visualized using the enhanced chemiluminescence reagent (Cytiva, Tokyo, Japan) as described by the manufacturer. Target protein band densities were measured using ImageJ (NIH, Bethesda, MD, USA).

2.5. Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA, RRID:SCR_002798). Treatment group means were compared using one-way analysis of variance followed by post hoc Tukey's tests for pair-wise comparisons.

3. Results

3.1. Live-cell imaging using ThT

We initially investigated the utility of ThT for staining structures aside from aggregated proteins in live cells. Surprisingly, we observed several intense round signals in the nucleus of mouse hippocampal HT22 cells following incubation in medium plus ThT for 30–60 min (Fig. 1). Addition of ThT to the extracellular medium also stained the cytoplasm in a mesh-like pattern. A similar staining pattern was observed in human embryonic kidney (HEK) 293 cells following incubation (Fig. 1). The distinctive round morphology and location of these structures suggested that they were nucleoli, which is plausible as ThT has been shown to bind RNA in the nucleolus.

3.2. ThT-positive puncta in the nucleus correspond to nucleoli

To determine if these ThT-positive round structures are nucleoli, we performed double staining on fixed cells using ThT and the fluorescent RNA-staining probe Nucleolus Bright Red. Indeed, green ThT and Nucleolus Bright Red signals overlapped (merged yellow), confirming the identity of these structures as nucleoli (Fig. 2) and consistent with

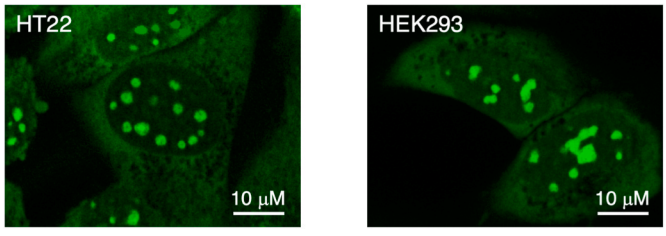

Fig. 1. Fluorescence imaging of nucleoli in cultured HT22 and HEK 293 cells using thioflavin T (ThT) as an RNA stain. Cells seeded on 35-mm glass-bottom dishes were incubated with ThT (5 μM) for 1 h, and live imaging was performed using a Keyence BZ-X810 microscope equipped with an optical sectioning algorithm system (Tanei et al., 2019).

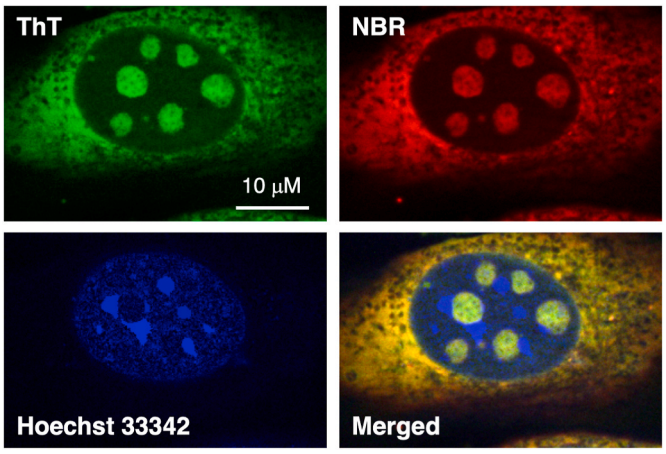

Fig. 2. Identification of Thioflavin T (ThT)-positive puncta as nucleoli in fixed HT22 cells by triple staining. Fixed cells were stained with the indicated combination of ThT (5 μM, green), Nucleolus Bright Red (5 μM, red), and Hoechst 33342 (10 μg/mL, blue) for 30 min. ThT staining overlapped with Nucleolus Bright Red staining (merged image) but not with the double-stranded DNA stain Hoechst 33342. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the known affinity of ThT for RNA in the nucleolus. These results indicate that ThT is suitable for imaging the nucleolus in both live and fixed cells. Alternatively, we observed little staining overlap between ThT and Hoechst 33342, a cell-permeable nucleic acid stain that binds to the minor groove A-T rich regions of double-stranded DNA, suggesting that ThT exhibits selectivity for RNA.

3.3. Imaging of ThT with organelle-specific fluorescent probes

To further assess the specificity of ThT staining, we examined the colocalization of ThT signals with those of the mitochondrial fluorescent probe MitoMM2 (Maeda et al., 2021), the lysosomal probe LysoBrite Red, and the endoplasmic reticulum (ER) probe ER tracer Red. Cytoplasmic ThT-positive signals showed little overlap with mitochondrial, lysosomal, or ER staining (Fig. 3), suggesting that the cytoplasmic mesh pattern of ThT signals mainly reflects the distribution of RNA molecules.

3.4. Oxidative stress reduces the number of nucleoli

Nucleoli are highly enriched in RNAs, which are among the most sensitive biomolecules to oxidative damage (Kong and Lin, 2010; Tanaka and Chock, 2021). We therefore speculated that nucleolar RNAs would be oxidized by ferroptosis and that the associated changes could be detected by ThT staining. Treatment of HT22 cells with the known ferroptosis inducers erastin and RSL3 (Dixon et al., 2012) significantly reduced the number of ThT-positive puncta, suggesting loss of nucleolar integrity (Fig. 4A). Similarly, the endogenous pro-oxidant hydrogen peroxide, which generates highly potent hydroxyl radicals in the presence of iron and thus may be a critical mediator for ferroptosis, reduced the number of ThT-positive puncta at a known cytotoxic concentration (0.75 mM, Fig. S1). The selective damage of nucleolar RNAs is confirmed by the Western blotting of nucleophosmin (B23/NPM1), which is localized in the nucleolus (Michalik et al., 1981). The protein levels of nucleophosmin were not affected in cells treated with RSL3, despite a reduction in GPX4, a ferroptosis marker (Fig. 4B). In contrast, the ER stress inducers thapsigargin and tunicamycin at effective concentrations had no measurable effects on nucleolar morphology or number (Fig. 4C). These results suggest that nucleolar RNAs are sensitive to oxidative stress and ferroptosis, although not to ER stress, and that ferroptosis may drive cell death in part by damaging nucleolar RNAs and thereby impairing nucleolar function.

4. Discussion

Live-cell imaging of the nucleolus can be performed using

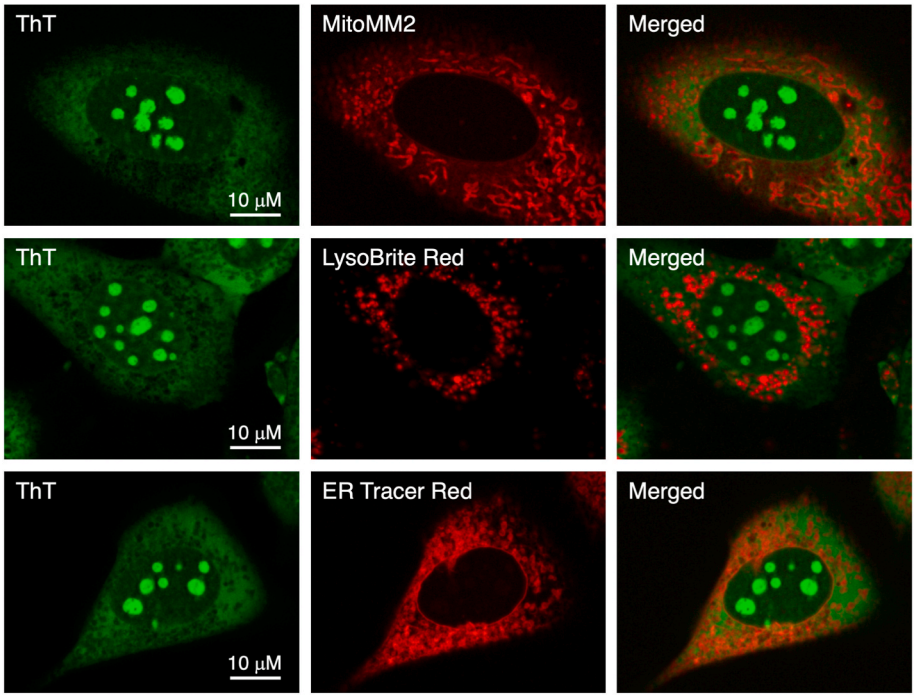

Fig. 3. Thioflavin T (ThT) did not stain mitochondria, lysosomes, or endoplasmic reticulum. Cells were doubled stained for 1 h at 37 °C with ThT (5 μM) and the mitochondria-specific red fluorescent dye MitoMM2 (0.5 μM) in the culture medium; or the lysosomal stain LysoBrite Red (AAT Bioquest, Sunnyvale, CA, USA), or the ER-specific ER Tracer Red (AAT Bioquest), according to the manufacturer's protocol. No substantial staining overlap was observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

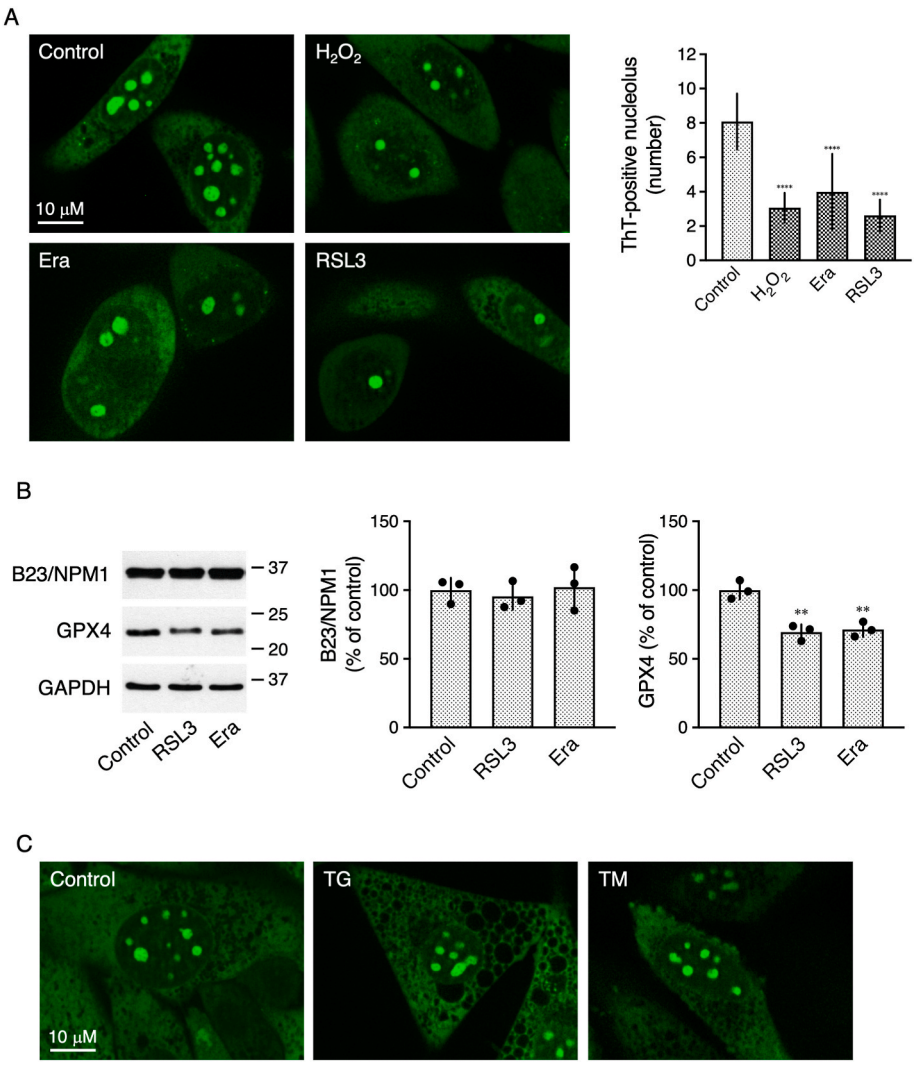

Fig. 4. Ferroptosis but not ER stress reduced the number of ThT-stained puncta in HT22 cells. (A) HT22 cells were treated with 0.75 mM hydrogen peroxide for 2 h, and 1 μM erastin for 9 h, or 0.5 μM RSL3 for 5 h to induce ferroptosis (Kato et al., 2023). Subsequently, the cells were stained with ThT for an additional 1 h. Representative fluorescence images are shown on the left, and quantification of the number of ThT-positive nucleoli on the right (as mean ± SD; n = 8–13). Statistical significance is denoted as ****P < 0.0001 vs. control. (B) The protein levels of B23/NPM1 and GPX4 were determined using Western blot analysis as described in the Materials and methods. Left: Representative immunoblots of typical data are presented. Right: The band intensity was quantified using Image J. The data are presented as the mean ± S.D. of three independent cultures. **P < 0.01 compared with the control. (C) HT22 cells were treated with 50 nM thapsigargin (TG) for 48 h or 0.1 μg/mL tunicamycin (TM) for 48 h to induce ER stress (Hasegawa et al., 2022), followed by staining with ThT for an additional 1 h.

fluorescent probes that specifically stain RNA. Nucleolus Bright Red, Nucleolus Bright Green, and SYTO RNA select are recommended for fixed samples, while PhenoVue 512 Nucleic acid stain (red and green) can be used for both live and fixed cells. However, the latter is not applicable for red/green double staining of proteins enriched in the nucleolus. In addition, several fluorescent naphthalimide derivative have been developed to stain ribosomal RNA in live cells (Cao et al., 2019). Compared with these reagents, however, ThT is less expensive. Also, ThT is soluble in both water and ethanol, highly cell permeable, and emits strong fluorescence upon binding to RNAs but not in RNA-free solvents. These properties allow for nucleolar imaging without the need for a medium change, making it applicable for damaged cells that easily detach from the culture plate.

Ferroptosis is a form of regulated cell death characterized by iron-dependent lipid peroxidation (Dixon et al., 2012) and exhibiting morphological and biochemical features distinct from other forms of cell death such as apoptosis and necrosis. Ferroptosis is triggered by the accumulation of reactive oxygen species (ROS) and lipid peroxides, and results in damaged mitochondria, lysosomes, and cell membranes (Stockwell, 2022). For instance, mitochondria exhibit iron-dependent disruption of membrane integrity and metabolic function during ferroptosis (Jelinek et al., 2018). In the current study, ThT staining revealed a selective decrease in nucleolar RNAs in both ferroptosis-induced and hydrogen peroxide-treated cells. We previously reported that levels of intracellular superoxide and hydrogen peroxide increased in ferroptosis-induced HT22 cells (Hirata et al., 2022). These results suggest that increased intracellular ROS production contributes to the dramatic reduction of nucleolar RNA. Taken together, we demonstrate for the first time that the ferroptosis induces nucleolar stress, likely due to the susceptibility of RNA molecules to ROS generated in the presence of iron, such as hydroxyl radicals from hydrogen peroxide. Given that neuronal RNA oxidation has been suggested to represent one of the fundamental abnormalities in age-associated neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Nunomura et al., 2002), oxidative damage of RNA molecules by ferroptosis might be involved in the pathogenesis of these disorders.

5. Conclusions

We demonstrate that the fluorescent reagent ThT can be used to stain nucleoli with high efficiency and specificity in both living and fixed cells without excessive protein aggregates. Moreover, ThT is a cost-effective and readily available reagent that does not require a medium change, making it suitable for comparing or evaluating significant impact on nuclear rRNA. Additionally, we report that ferroptosis, but not ER stress, can reduce the number of nucleoli as revealed by ThT staining. These findings suggest that nucleolar RNA molecules are susceptible to damage by ferroptosis and that this nucleolar damage may contribute to diseases involving ferroptosis.

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CRediT authorship contribution statement

Yoko Hirata: Writing – original draft, Methodology, Investigation. **Hiroshi Takemori:** Methodology, Resources. **Kyoji Furuta:** Methodology, Investigation. **Yuji O. Kamatari:** Methodology, Investigation. **Makoto Sawada:** Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphar.2024.100196.

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