



Architecture-driven design of near-infrared probes for bioimaging of senescence in biological systems

Stavroula G. Kyrkou^{a,1} , Despoina D. Gianniou^{b,1} , Dimitrios A. Diamantis^a,
Aimilia D. Sklirou^b , Eleni Patsea^c, Vassilis Zoumpourlis^d, Eleftherios Paraskevas Tzakos^b ,
Demeter Tzeli^{e,f} , Ioannis P. Trougakov^b, Andreas G. Tzakos^{a,*}

^a Department of Chemistry, Section of Organic Chemistry and Biochemistry, University of Ioannina, Ioannina 45110, Greece

^b Department of Cell Biology and Biophysics, Faculty of Biology, National and Kapodistrian University of Athens, Athens 15784, Greece

^c Department of Pathology, Metropolitan Hospital, Cholargos 18547, Greece

^d Biomedical Applications Unit, Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece

^e Laboratory of Physical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, Athens 157 84, Greece

^f Theoretical and Physical Chemistry Institute, National Hellenic Research Foundation, 48 Vassileos Constantinou Ave., Athens 116 35, Greece

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ABSTRACT

Cellular senescence contributes to aging, tissue remodeling, and various pathologies including cancer and fibrosis, motivating the development of sensitive and selective tools to detect senescent cells in complex biological settings. Here we report SenNIR, a lysosome-targeted near-infrared (NIR) fluorescent and colorimetric probe engineered for selective imaging of senescence. SenNIR integrates a senescence-associated β -galactosidase (SA- β -gal)-responsive trigger, a lysosomal localization element, and a dicyanomethylene-4H-pyran fluorophore. Upon SA- β -gal activation, the lead probe, SenNIR-1 (*meta-ether* linkage), displays a NIR fluorescence turn-on response (~ 17 -fold enhancement), enabling sensitive detection of senescent cells. To interrogate how molecular architecture governs function, we evaluated structural analogues altering the connectivity and tethering topology of the modules. Strikingly, these systematic variations revealed that linker identity and the substitution position of the lysosomal targeting unit strongly regulate the probe's photophysical response. The effect arises from architecture-dependent control of excited-state energetics, specifically the relative ordering of charge-transfer versus locally excited states, which is reorganized upon enzymatic unmasking to produce an efficient fluorescence turn-on. Transitioning to an *ortho-amide* linkage (SenNIR-2) maximized the electronic coupling, yielding a > 100 -fold NIR turn-on, whereas an *ortho-ether* linkage (SenNIR-3) produced a ~ 9 -fold enhancement but at deeper NIR wavelengths. All SenNIR probes showed no detectable cytotoxicity in normal human skin fibroblasts and robustly differentiated proliferating from prematurely senescent cells. Probe performance and SA- β -gal specificity were further validated in *ex vivo* models. Collectively, these results demonstrate that not only the choice of functional modules but also their mode of assembly is decisive for diagnostic performance, providing design rules for next-generation senescence probes.

1. Introduction

Aging is a multifactorial, time-dependent process characterized by a gradual decline in physiological resilience and loss of organismal homeodynamics [1]. It is the major risk factor for numerous diseases including type 2 diabetes, cardiovascular diseases, cancer, neurodegeneration and other vulnerabilities [2–6]. The major hallmarks of

aging, among others, include epigenetic modifications, genomic instability, telomere attrition, mitochondrial dysfunction as well as increasing rates of cellular senescence [1]. Cellular senescence can be triggered by either telomere shortening (replicative senescence, RS) or in normal cells with long telomeres as a response to various damaging stimuli (stress-induced premature senescence, SIPS) including DNA damage, reactive oxygen species (ROS), aberrant activation of

* Corresponding author.

E-mail address: atzakos@uoi.gr (A.G. Tzakos).

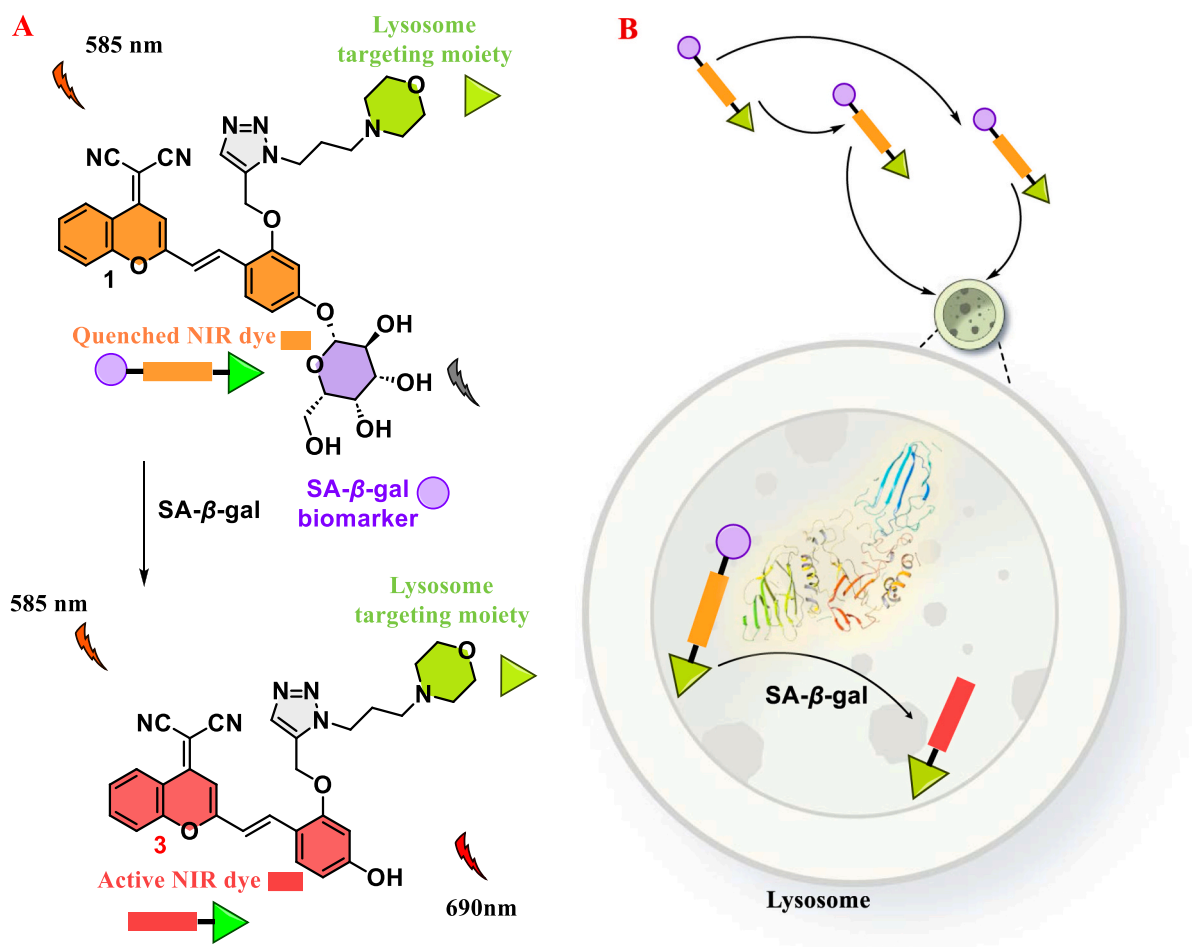
¹ Equal contribution

Table 1

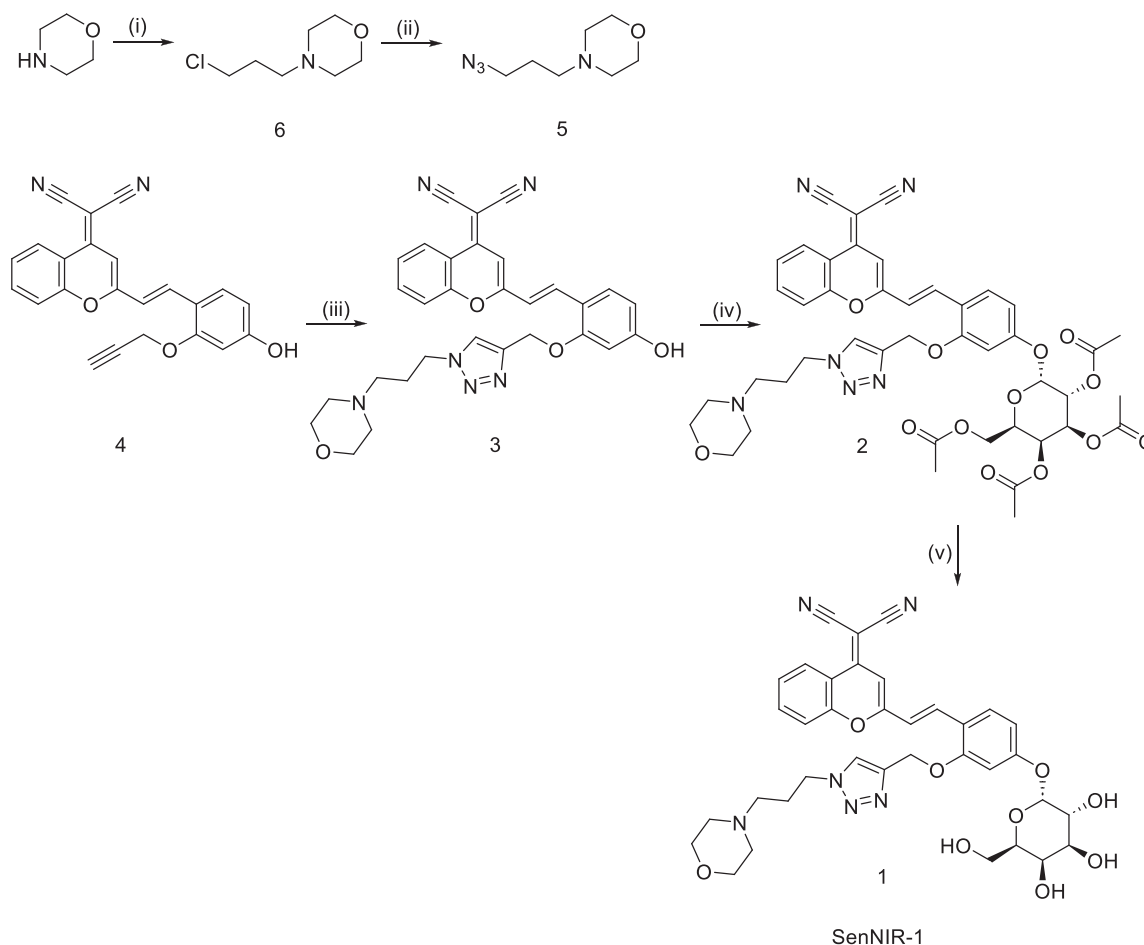
Comparative overview of recent SA- β -gal responsive probes and sensors, highlighting their key photophysical properties, structure-activity relationships, and biological application levels.

Sensor Type	Key Features	Photophysical Response in Solution (Turn-on)	Application Level	Under mediated responsiveness or Structure-Property Relationships	Ref
NIR fluorescent probes	Deep-tissue imaging, large Stokes shift, high sensitivity	~4-fold nr	<i>In vitro</i> / <i>In vivo</i>	no	[30–32]
Self-immobilizing / covalent-labeling probes	Long-term retention inside senescent cells; prevents probe leakage	6-fold	<i>In vitro</i>	no	[33]
Ratiometric fluorescent probes	Internal self-calibration; improves quantification	25-fold and 13-fold (in cellular level)	<i>In vitro</i> / <i>In vivo</i>	no	[34,35]
Species-selective probes	Distinguish human SA- β -gal from bacterial β -gal	nr ^a	<i>In vitro</i>	yes	[36]
Logic-gated / multi-dimension probes	Dual sensing (e.g., β -gal + pH/ROS) improves selectivity	nr	<i>In vitro</i> / <i>In vivo</i>	no	[37,38]
Two-photon probes	High-penetration imaging in tissues and plants	Not explicitly reported (16-fold increase observed between 0.5 and 1 min in cells)	<i>In vitro</i>	no	[39]
Far-red/photoablation probes	Imaging + senolytic phototherapy	95-fold	<i>In vitro</i>	no	[40]
Lysosome-targeted probes (non-fluorescent & fluorescent)	drug-based galactoside prodrugs (non-Fluorescent)	nr	<i>In vitro</i> / <i>In vivo</i>	yes	[41]
Dual-Function (Targeting & Cleavable) Probes	~4.3-fold fluorescence enhancement determined at pH 4.0	~4.3-fold and nr	<i>In vitro</i>	no	[42,43]

^a nr- not reported.



Scheme 1. SenNIR-1 is a NIR fluorescent probe that selectively accumulates in senescent cells and is activated through its interaction with SA- β -gal. (A) The structural architecture of SenNIR-1 and its turn-on mechanism triggered by SA- β -gal. (B) Schematic illustration of lysosome-targeted delivery and enzymatic activation of SenNIR-1 for NIR fluorescence imaging in senescent cells.



Scheme 2. Synthetic route for compound **SenNIR-1** where all intermediates are depicted. The details of the synthesis are pinpointed in the sections below. Reagents and solvents: **i)** 3-iodo 1-chloro propane, reflux, toluene, 4 h, $\alpha = 43.5\%$ **ii)** NaN_3 , $\text{H}_2\text{O}/\text{MeOH}$, reflux 15 h, $\alpha = 85\%$ **iii)** $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, DMF, 25°C , $\alpha = 62.5\%$ **iv)** tetra-O-acetyl- α -D-galactopyranosyl-1-bromide, TEA, 25°C , ACN, 15 h, $\alpha = 43.2\%$ **v)** MeONa, MeOH, 25°C , 6 h, $\alpha = 62.6\%$.

oncogenes [7], etc. Whereas cellular senescence (RS or SIPS), a state of highly stable cell cycle arrest, is considered an intrinsic tumor suppression mechanism, it appears that age-related accumulation of senescent cells in the human body can promote tumorigenesis e.g., via their senescence-associated secretory phenotype (SASP); a phenotype where senescent cells secrete high levels of immune modulators, inflammatory cytokines and proteases promoting thus tumor growth [8, 9]. Therefore, although cellular senescence is an adaptation “aiming” to interrupt the proliferation of damaged and/or stressed cells [7,10–13], the gradual accumulation of senescent cells in aged tissues leads to tissue/organ dysfunction and accelerates aging [14,15].

Reliable detection of senescent cells in biological systems remains challenging because senescence is heterogeneous and context-dependent. A great number of biomarkers and phenotypic characteristics have been developed to monitor cellular senescence including measurement of SASP-related cytokines, the expression of cell cycle inhibitors and/or tumor suppressors (e.g., p16, p21 or p53), as well as the detection of β -galactosidase (β -gal) in senescent cells [16–19]. β -gal is a lysosomal hydrolase that catalyzes the cleavage of the galactose moiety and converts the lactose into monosaccharide. Its optimal enzymatic activity in young replicating human cells ranges from pH 4–4.5; yet, by using certain biochemical reactions at pH 6, the elevated lysosomal β -gal activity (SA- β -gal) that is detectable specifically in senescent (RS or SIPS) cells can be used to discriminate these cells from young replicating human cells [20,21]. Indeed, compared with proliferating cells, senescent cells display an expanded lysosomal compartment, reflected by increased lysosomal number, size, and mass, elevated

levels of lysosomal hydrolases, including SA- β -gal and a relative increase in lysosomal pH [22–25]. Thus, it is not surprising that in order to monitor β -gal activity in different physiological conditions, including senescence and aging, or cancer, a great number of diagnostics have been developed [26–29]. Table 1 summarizes the representative classes of senescence probes developed to date, highlighting their primary features [30–40]. As illustrated, while many platforms achieve proof-of-concept sensing primarily in live cells, there remains a lack of systematic studies investigating molecular architecture, specifically the connectivity and tethering topology of the trigger, the targeting group, and the fluorophore. Most existing methodologies do not systematically explore these topochemical parameters to unlock high sensitivity (e.g., >100-fold enhancement) and robust validation in *ex vivo* aged tissue models. Addressing this critical gap requires a comprehensive, architecture-driven approach.

Optical imaging offers a compelling route for senescence detection because it is sensitive, operationally simple, and compatible with live-cell and tissue-level measurements, serving as a powerful tool for intracellular pathway visualization [44–46]. Consequently, numerous fluorescent probes have been developed for β -gal visualization in cells [47–53]. However, relatively few probes emit in the near-infrared (~ 650 – 900 nm), a spectral window that reduces background autofluorescence and enables improved tissue penetration compared with visible-emitting dyes [31,54–57]. Moreover, although SA- β -gal is lysosomal, the availability of activatable NIR probes that combine SA- β -gal responsiveness with reliable lysosomal targeting [41,42], a critical feature for specific subcellular tracking, remains limited [58]. Beyond

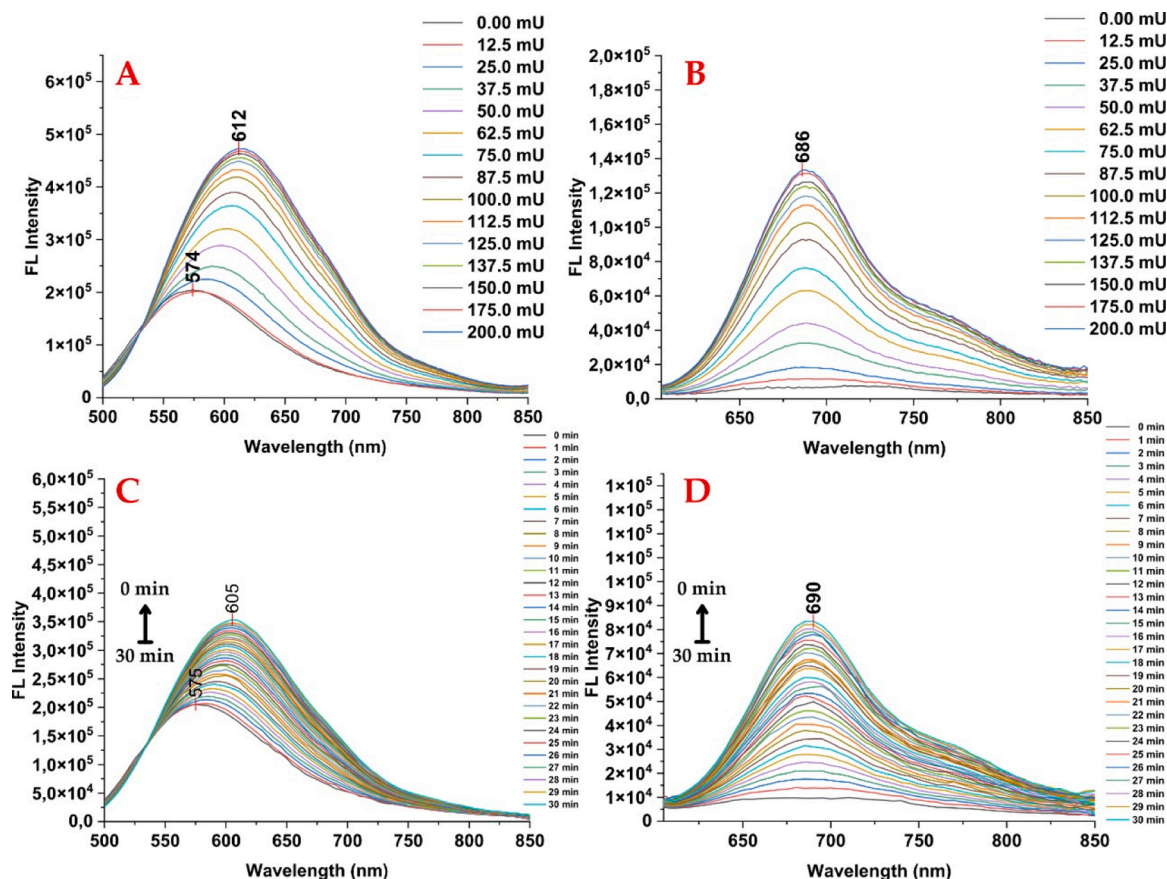


Fig. 1. Spectral properties of SenNIR-1 (10 μ M) in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) (A), (B) Normalized Fluorescent intensity of SenNIR-1 upon excitation at 465 and 585 nm, respectively in the presence of different concentrations of β -gal, (C), (D) Time dependence of emission spectra (0–30 min) of SenNIR-1 in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 465 and 585 nm respectively, following treatment with 0.1 U of β -gal.

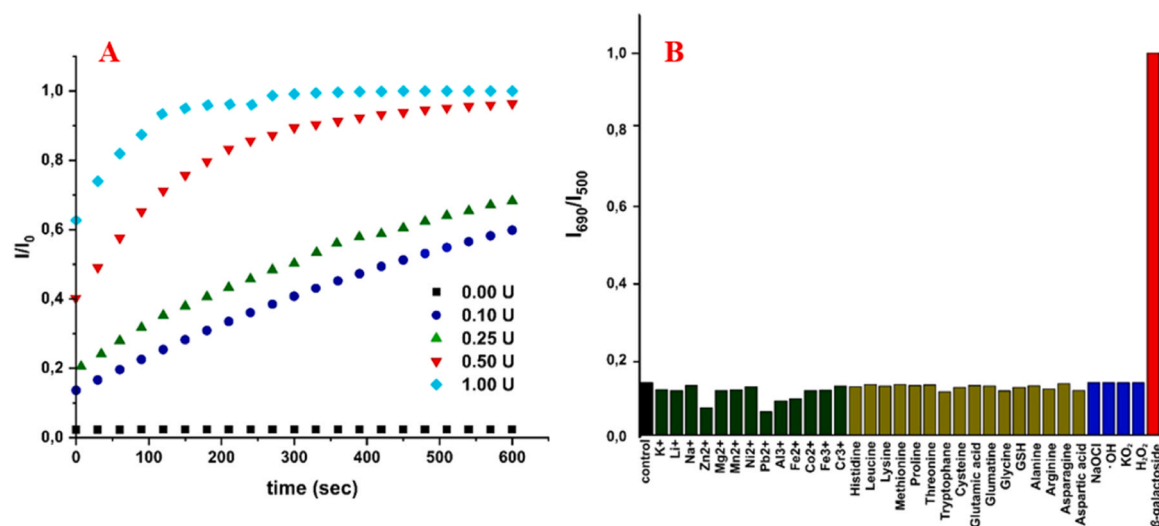


Fig. 2. A) Kinetics of the fluorescence signal at 690 nm of SenNIR-1 (10 μ M) in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 585 nm in the absence or presence of different of β -gal concentrations. B) Fluorescence ratio (I_{690}/I_{500}) responses of SenNIR-1 (10 μ M) to various analytes in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 465 nm.

spectral constraints, many platforms focus on proof-of-concept sensing in cells with limited evaluation in tissues. Notably, recent advancements have introduced NIR chemosensors capable of performing analyte-triggered detection even in complex biological matrices, demonstrating their robustness for applications in both practical and living-cell environments [59]. Consequently, there remains a lack of

systematic studies investigating how molecular architecture (the connectivity and tethering of the trigger, targeting group, and fluorophore) dictates overall performance. This is a practical limitation for probe engineering because the photophysical response of donor - acceptor NIR dyes is highly sensitive to substituent position, linker identity, and the resulting excited-state landscape. Indeed, recent work has highlighted

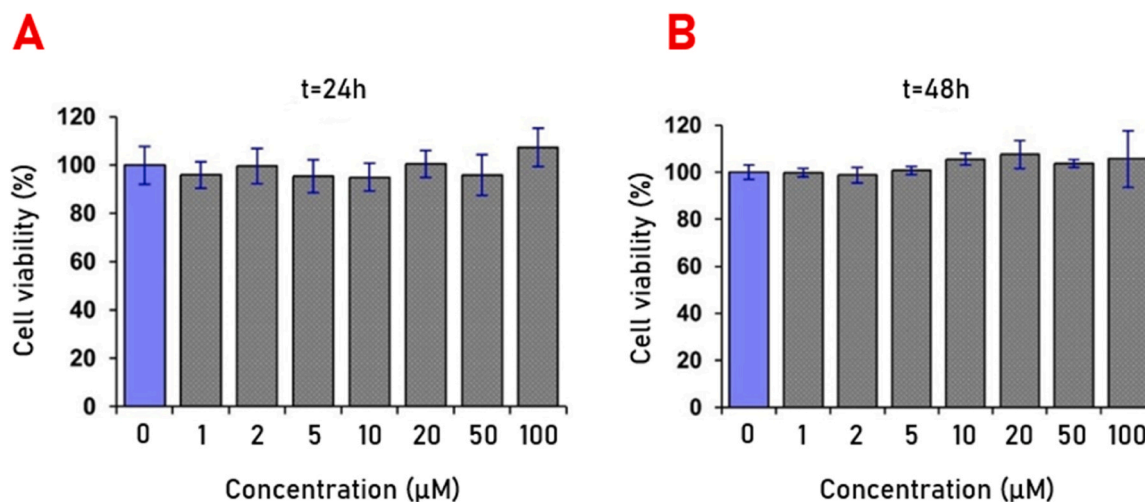


Fig. 3. SenNIR-1 does not exert any cytotoxic effect in normal human skin fibroblasts. Relative (%) survival levels (MTT assay) of BJ skin fibroblasts incubated with the indicated concentrations of the SenNIR-1 for 24 (A) and 48 (B) h. Control samples values (untreated cells) were set to 100%; bars, $\pm$ SD ($n \geq 3$).

that fine-tuning molecular symmetry and substituent arrangements is fundamental for modulating the excited-state dynamics and optimizing the performance of NIR sensing platforms [60].

Herein, we present **SenNIR**, a colorimetric and activatable NIR fluorescent platform engineered for selective detection of senescent cells in biological systems. **SenNIR** combines three functional modules: a D-galactopyranosyl trigger that is cleaved by senescence-associated SA- β -gal, a morpholine unit to promote lysosomal accumulation, and a dicyanomethylene-4H-pyran (DCM) fluorophore to provide NIR readout. Enzymatic activation by SA- β -gal restores the donor capability of the phenolic unit and re-establishes intramolecular charge transfer (ICT), producing a pronounced red shift in the optical response and a strong fluorescence turn-on in the NIR window. In cells, **SenNIR** is cytocompatible in normal human BJ fibroblasts and discriminates proliferating from SIPS cells by confocal imaging, while *ex vivo* tissue sections from young *versus* aged mice confirm age-dependent signal enhancement.

To interrogate how the architecture of the probe controls its function, we evaluated structural variants that utilize the same trigger, targeting, and fluorophore components but differ in their linker topologies. Remarkably, these changes translated into order-of-magnitude differences in diagnostic output, ranging from single-digit/low-tens fold enhancement to $\sim 10^2$ -fold NIR turn-on upon enzymatic activation, establishing linker architecture as a previously underappreciated driver of senescence-imaging performance. The observed differences are consistent with an architecture-dependent modulation of the excited-state landscape and donor-acceptor coupling. Upon deglycosylation, electron density is efficiently relayed from the liberated phenolate through the conjugated π -system to the DCM acceptor, while the targeting linker can further tune this process through inductive/resonance effects and different degrees of intramolecular hydrogen bonding and preorganization. Collectively, these results show that **SenNIR** performance is not dictated solely by the choice of functional modules but critically by their mode of assembly, providing actionable design rules for next-generation senescence probes and possibly as a platform for senolytic or theranostic development.

2. Materials and methods

2.1. Chemicals

All commercially available chemicals were used without further purification. All reactions were performed with dry solvents under anhydrous conditions, unless otherwise noted. Methylene chloride

(CH₂Cl₂), ethyl acetate (EtOAc) and piperidine were purchased in anhydrous form and used without further purification. Air- and moisture-sensitive liquids were transferred via syringe. Organic solutions were concentrated by rotary evaporation at 36 °C. Column chromatography was performed with Acros Organics silica gel 60 (230–400 mesh). Thin layer chromatography (TLC) was performed with pre-coated Merck silica gel 60 F₂₅₄ plates. TLC plates were visualized by exposure to ultraviolet light (UV) and/or submersion in aqueous potassium permanganate solution (KMnO₄/H₂SO₄). ¹H NMR spectra were recorded either on a 250 MHz or 400 MHz or 500 MHz Bruker Avance FT NMR spectrometer. β -galactosidase from *Aspergillus oryzae* was purchased from Sigma Aldrich.

2.2. UV-Vis spectroscopy

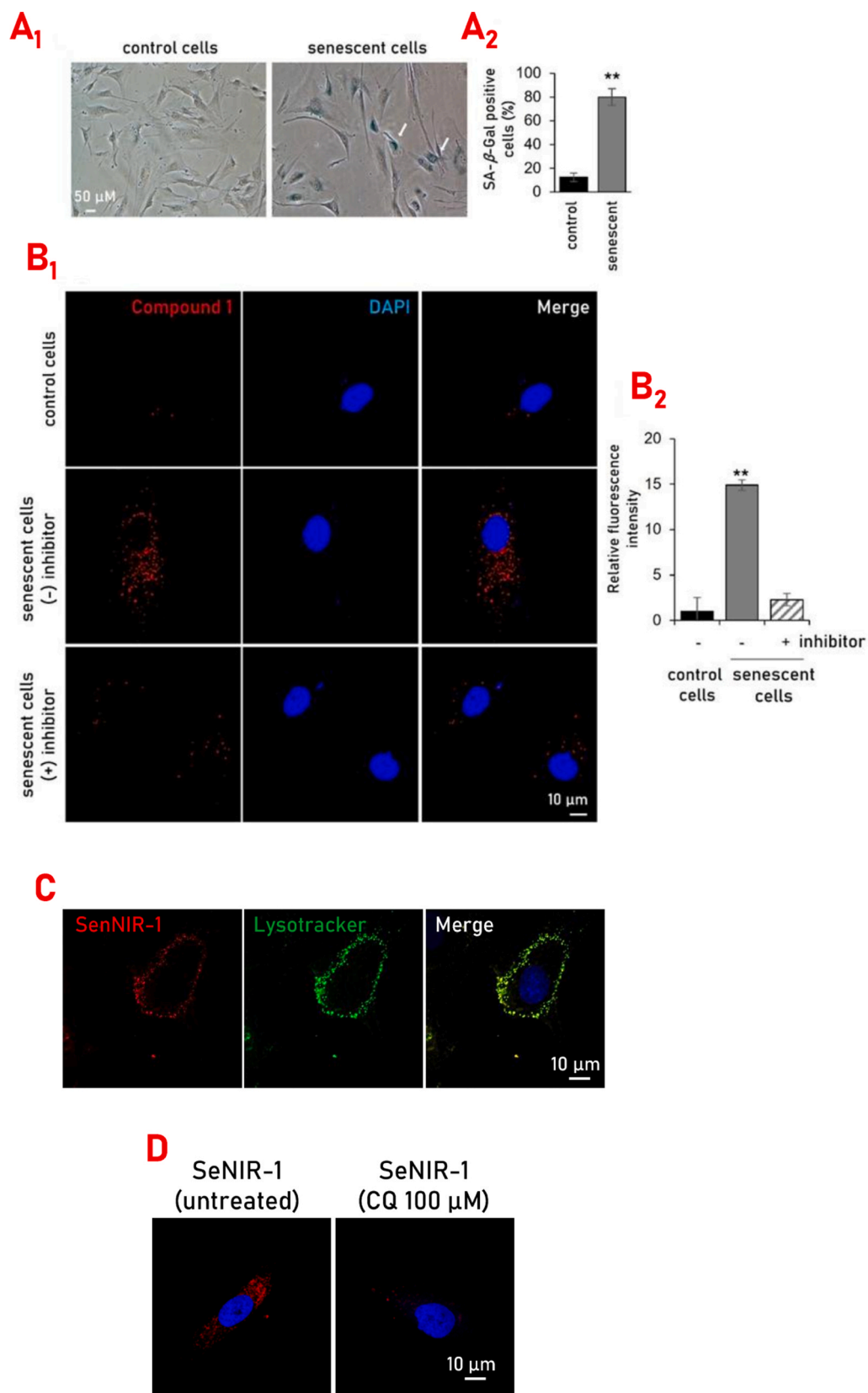
UV-Vis spectra were recorded with an Edinburg DS5 spectrophotometer in a 1 cm quartz cell at 37 °C. Typical titration experiments were performed by sequential additions of β -gal to the same 3 mL sample solution (10.0 μ M) in a quartz cuvette.

2.3. Fluorescence spectroscopy

Fluorescence spectra were recorded with an Edinburg FS5 spectrophotometer (slit=10, excitation at 465/585 nm) in a 1 cm quartz cell at 37 °C. Typical titration experiments were performed by sequential additions of β -gal to the same 3 mL sample solution (10.0 μ M) in a quartz cuvette.

Fluorescence lifetime decays were recorded using a time-correlated single-photon counting (TCSPC) system. The samples were excited using a pulsed light source with a pulse width of 100 ns. The data were acquired over 2048 channels with a time resolution of 20 ns per channel. For the SenNIR-1 probe, a specific optical filter (filter 5) was employed for emission collection, while for SenNIR-2 and SenNIR-3, a filter 6 setting was used. All measurements were conducted at room temperature in their respective phosphate buffer solutions. The decay curves were analyzed to determine the fluorescence lifetimes, ensuring optimal signal-to-noise ratios for accurate fitting.

The fluorescence quantum yields (Φ) of the probes were determined in aqueous media via the comparative method. Curcumin was utilized as the standard reference, possessing a known quantum yield of 0.0110 (1.10%) in water [61,62]. The quantum yields were calculated by comparing the integrated fluorescence emission intensities of the samples against those of the reference. To ensure measurement precision, all spectroscopic data were acquired at a strictly controlled temperature of



(caption on next page)

Fig. 4. SenNIR-1 visualizes SA- β -gal activity in senescent cells. (A₁) Representative light field images following SA- β -gal cytochemical staining of control BJ cells or cells treated (three exposures of 48 h each) with 300 μ M H₂O₂. (A₂) Relative (%) number of SA- β -gal positive cells following treatment of cells exactly as in (A₁). CLSM visualization (B₁) and quantitation of intensity (B₂) of SenNIR-1 fluorescence in control and premature senescent (H₂O₂ treated) cells. Cells were cultured on coverslips and incubated with 10 μ M of SenNIR-1 for 1 h, upon or without pretreatment with the β -gal inhibitor (D-galactose; 1 mM) for 1 h. (C) CLSM visualization of senescent BJ cells with LysoTracker (Green) and SenNIR-1 (red). (D) CLSM visualisation of senescent BJ cells incubated with SenNIR-1 (red) following pretreatment with CQ (100 μ M for 2 h). Cells were fixed, stained with DAPI (blue) and mounted on slides before observation under a CLSM. Control samples values (untreated cells) were set to 1 (B₂). Bars $\pm$ SD. ** $p < 0.01$. Bars, in (A₁) 50 μ m and in (B₁, C D) 10 μ m.

20 °C. The samples were excited at 425 nm, with both excitation and emission slits adjusted to 5 nm to maintain consistent optical resolution throughout the measurements.

2.4. Detection limit

The detection limit was determined from the fluorescence titration data based on reported methods [63–65]. The fluorescence spectrum of SenNIR-1 was measured five times, and the standard deviation of the blank measurement was extracted. To calculate the slope, the fluorescence intensity data at 690 nm was plotted against the concentration of β -galactosidase. Similarly, the fluorescence spectra for SenNIR-2 and SenNIR-3 were recorded under identical conditions ($n = 5$) to determine the standard deviation of their respective blank measurements. For the slope calculation of SenNIR-2, the fluorescence intensity at 690 nm was utilized, whereas for SenNIR-3, the corresponding intensity data at 730 nm were plotted as a function of β -gal concentration. The detection limit was calculated with the following equation:

$$\text{LOD} = 3\sigma/k$$

Where σ is the standard deviation of blank measurement, and k is the slope between the fluorescence intensity versus the concentration of the analyte.

2.5. Mass spectrometry

2.5.1. Characterization

The Xevo G2 Q-TOF mass spectrometer was operated in positive electrospray ionization (ESI) mode for direct infusion analysis, employing full-scan MS across a mass range of 50–1200 m/z . The following optimized source conditions were applied to achieve maximum ion intensity: capillary voltage of 3 kV, sample cone voltage of 10 V, source temperature set to 120 °C, desolvation temperature of 250 °C, cone gas flow rate of 100 L h⁻¹, and desolvation gas (N₂) flow rate of 600 L h⁻¹. These settings ensured enhanced sensitivity and ionization efficiency during the analysis.

2.5.2. LC-MS validation of the enzymatic cleavage mechanism

To verify the SA- β -gal-mediated hydrolysis of the glycosidic bond and the release of the free fluorophore, an LC-MS kinetic assay was performed. The selected probe was incubated at a final concentration of 30 μ M with β -gal (*Aspergillus oryzae*, ~0.25 U per reaction, Sigma-Aldrich) in ddH₂O (pH 6.0) containing minimal organic co-solvent (2.5% ACN) to ensure probe solubility without inducing enzyme denaturation.

The reaction mixtures were incubated at 37 °C, and sample aliquots were processed at discrete time intervals (0, 5, 15, and 30 min). At each designated time point, enzymatic activity was quenched, and proteins were precipitated by the addition of a three-fold volume of cold ACN (–20 °C). A negative control lacking the enzyme was incubated in parallel for 30 min to confirm the chemical stability of the probe under the assay conditions. Following protein precipitation, the samples were centrifuged at 10,000 rpm for 10 min. The resulting supernatants were collected and subjected to LC-MS analysis.

LC-HRMS analyses were performed using a Waters ACQUITY UPLC system coupled to a Waters Xevo G2-XS Q-TOF mass spectrometer. Chromatographic separation was achieved on an ACQUITY UPLC BEH

C18 column maintained at 35 °C. The mobile phase consisted of water (Solvent A) and acetonitrile (Solvent B), both supplemented with 0.1% formic acid. A gradient elution program was applied at a constant flow rate of 0.200 mL/min, starting at 15% B (held for 10.00 min to reach 50% B), linearly increasing to 100% B at 11.00 min (held until 13.00 min), and returning to the initial conditions (15% B) at 13.50 min for equilibration until 15.00 min. The injection volume was set to 4.0 μ L. UV-Vis absorption was simultaneously monitored using a PDA detector, with continuous scanning from 210 nm to 750 nm. The mass spectrometer was operated in negative electrospray ionization (ESI⁻) mode. The acquisition mass range was set from m/z 100–1500. The key MS parameters were optimized as follows: capillary voltage at 1.0 kV, sampling cone voltage at 20.0 V, source offset at 80 V, source temperature at 120 °C, and desolvation temperature at 550 °C. MSE (data-independent acquisition) mode was utilized, simultaneously acquiring low-energy (precursor ion) and high-energy (fragmentation) data (collision energy ramp from 20 to 40 eV using argon). Leucine enkephalin was used as the lock mass reference compound.

2.6. Analytical HPLC

The product's purity was further evaluated by an Agilent analytical HPLC system equipped with an InfinityLab Poroshell 120 EC-C18 column (4.6 $\times$ 150 mm) and a diode array detector (DAD). Detection was carried out at both 254 nm and 475 nm. The analysis was performed using a gradient solvent system, from A (2%) and B (98%) to A (100%) and B (0%), where solvent A consisted of acetonitrile (ACN) with 0.1% formic acid, and solvent B was H₂O with 0.1% formic acid. This was followed by a 3-minute phase with A (100%) and B (0%), and finally, the system reverted to the initial conditions of A (2%) and B (98%) for an additional 2 min. The flow rate was maintained at 1.0 mL/min over a 20-minute period.

2.7. Cell Culture

Human foreskin fibroblasts (BJ cells) were purchased from the American Tissue Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) non-essential amino acids and were maintained in conditions of 5% CO₂, 95% humidity and 37 °C. In all experimental procedures applied, cells were subcultured by using a trypsin/EDTA solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). For all cell experiments, probes were diluted in serum-containing DMEM, so that the final DMSO content delivered to the cells was minimal (<0.1%).

2.8. Induction of premature senescence by hydrogen peroxide (H₂O₂) and SA- β -gal staining

For the induction of premature senescence, relatively early passage proliferating cells were exposed to serial short-term oxidative stress, as previously described [66]. Briefly, cells were treated with the sub-cytotoxic concentration of 300 μ M H₂O₂ (Merck KGaA, Darmstadt, Germany) (three exposures of 48 h each), while control cells were kept under the same culture conditions without H₂O₂.

SA- β -gal staining was performed, as reported before [67]. Cells grown on glass coverslips were washed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde in PBS for 10 min. Following

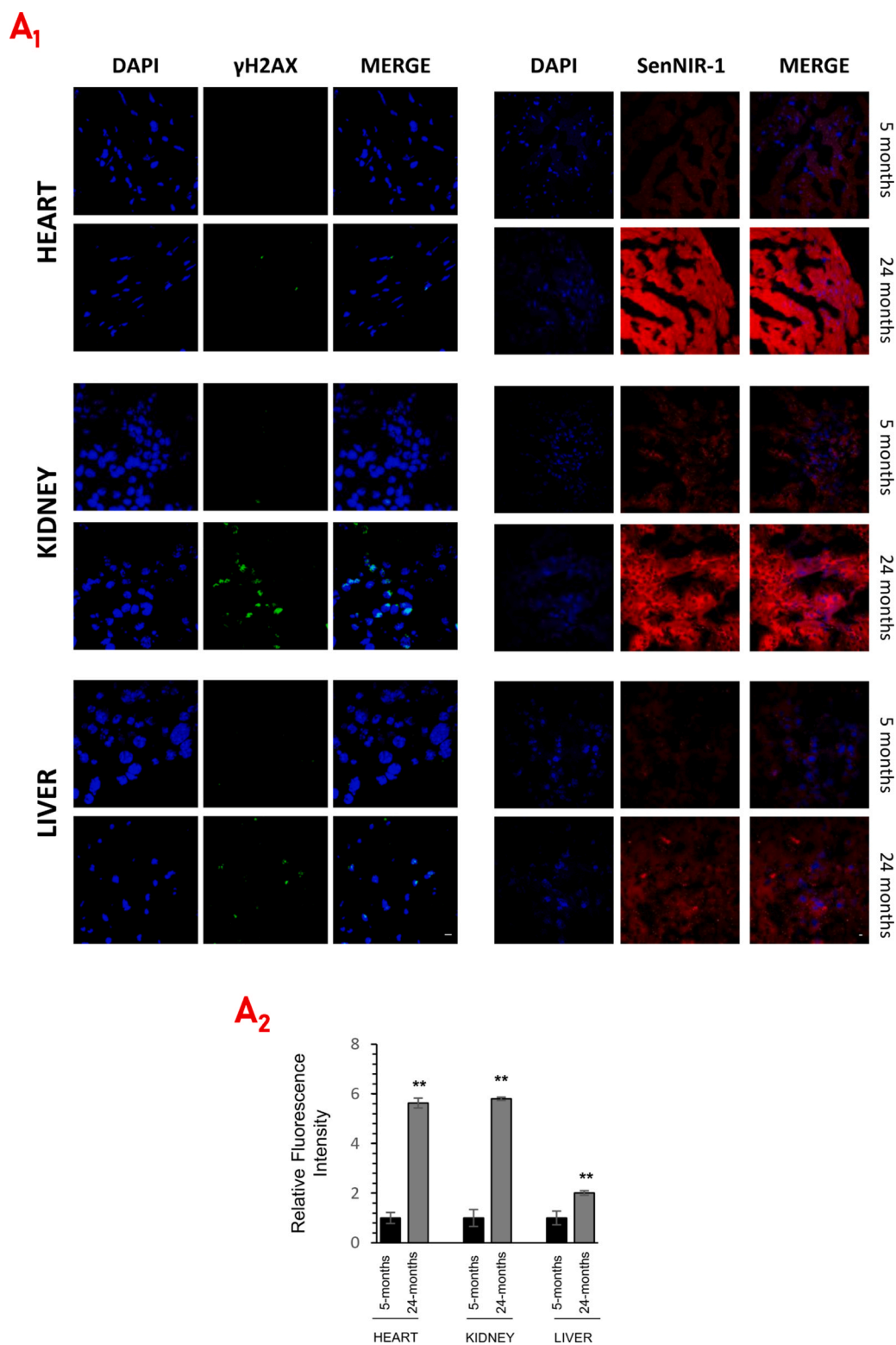
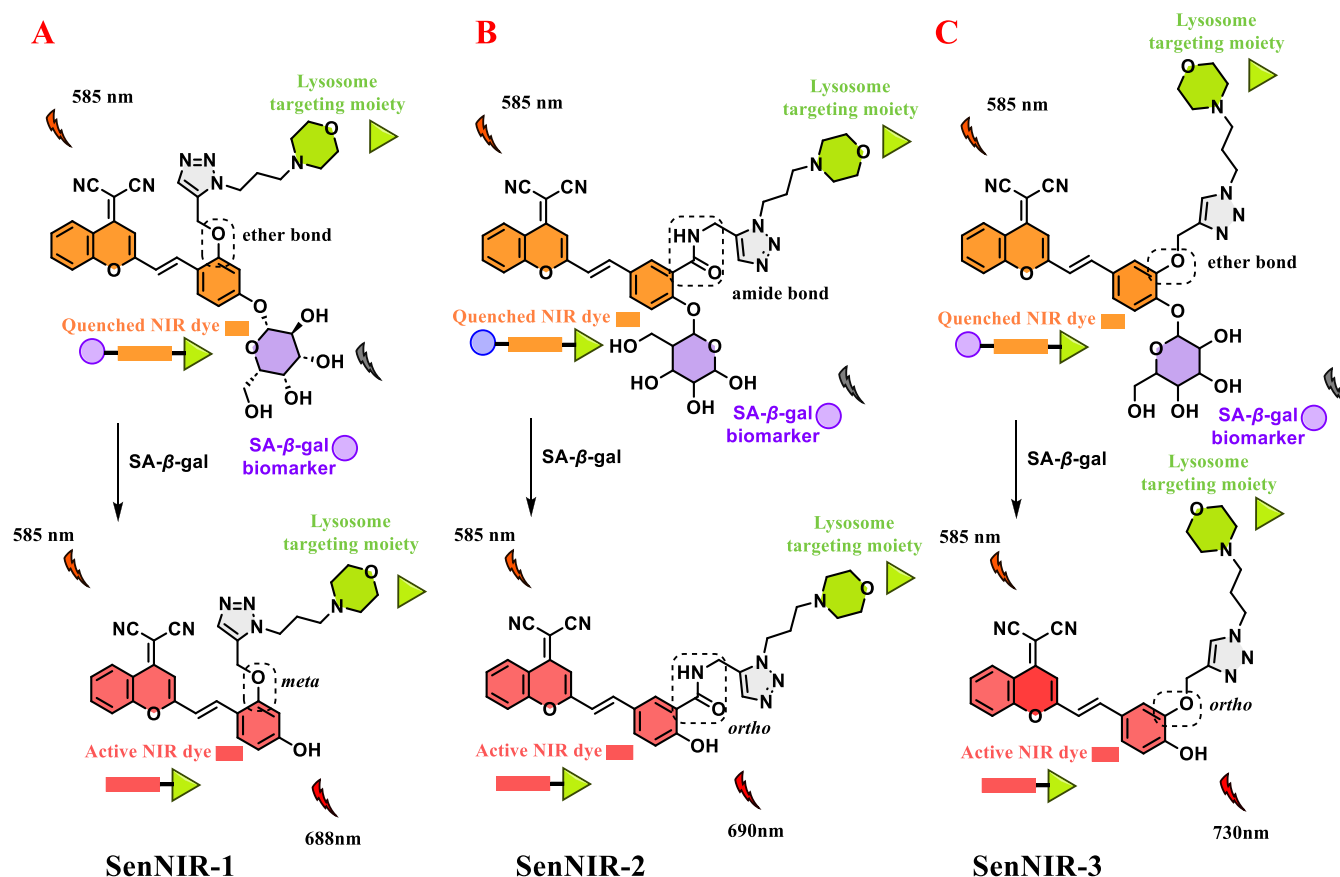


Fig. 5. (A₁) Representative images of heart, kidney and liver tissue sections from 5- and 24-month-old C57BL/6 J male mice incubated with **SenNIR-1** (20 μ M); samples were also immunostained for γ -H2AX (marker of DNA damage). Scale bar: 10 μ m. (A₂) Relative (%) fluorescence of **SenNIR-1** (20 μ M) in tissue sections from 5-, and 24-month-old C57BL/6 J male mice. Control (5months old) samples values were set to 1. Bars, $\pm$ SD; * $p < 0.05$; ** $p < 0.01$. $n = 5$ mice per age group; 6 sections per organ per animal.

fixation, cells were washed with PBS and were then stained (in the absence of CO₂) for 16 h at 37°C in staining solution [150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄[Fe(CN)₆]*3H₂O, 40 mM citric acid/sodium phosphate, pH 6.0 containing 1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactoside]. Samples were observed under phase

contrast optics in a TS-100F NIKON inverted microscope and at least 10 optical fields were used to score positively stained cells.



Scheme 3. Comparative senescence activation mechanisms of SenNIR analogues. The mechanism of action for **SenNIR-2** and **SenNIR-3** via SA- β -gal is illustrated in comparison to the lead probe, **SenNIR-1**. (A) Structural architecture of **SenNIR-1**, featuring the lysosomal targeting moiety linked via an ether bond at the *meta*-position relative to the molecular trigger. (B) Structural architecture of **SenNIR-2**, incorporating an amide bond linkage at the *ortho*-position relative to the trigger. (C) Structural architecture of **SenNIR-3**, featuring the lysosomal targeting moiety linked via an ether bond at the *ortho*-position relative to the trigger.

2.9. Cell viability assay

The effect of compounds on cell viability was estimated using the MTT assay, as previously reported [68]. Briefly, BJ cells plated in flat-bottomed 96-well microplates were treated with different concentrations of probes for 24 or 48 h. Afterwards, the medium was replaced by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) dissolved at a final concentration of 1 mg/mL in serum-free, phenol red-free medium. The formed formazan crystals were then dissolved by isopropanol and optical density was measured at 570 nm in a Spark® multimode microplate reader (Tecan Trading AG, Männedorf, Switzerland).

2.10. Confocal laser scanning microscopy (CLSM)

Cells grown on coverslips were incubated with 10 μ M (unless otherwise indicated) of probes for 1 h in an incubator of 5% CO₂, 95% humidity and 37 °C. Afterwards, they were fixed with 4% formaldehyde, counterstained with DAPI (Molecular Probes™/Thermo Fisher Scientific Inc., Waltham, MA, USA) and coverslips were then mounted in Mowiol. Visualization of samples was performed upon excitation of the probe at 543 nm using the Digital Eclipse C1 Nikon (Melville, NY, USA) CLSM equipped with a 60 $\times$ 1.40 NA DIC Plan Apochromat objective as well as the EZC1 acquisition and analysis software (Nikon). Quantification of fluorescence intensity in captured images was conducted by ImageJ software (National Institutes of Health, USA). For lysosomal colocalization studies, premature senescent (H₂O₂-treated) BJ cells were incubated with SenNIR-1 (10 μ M) for 1 h at 37 °C and co-stained with

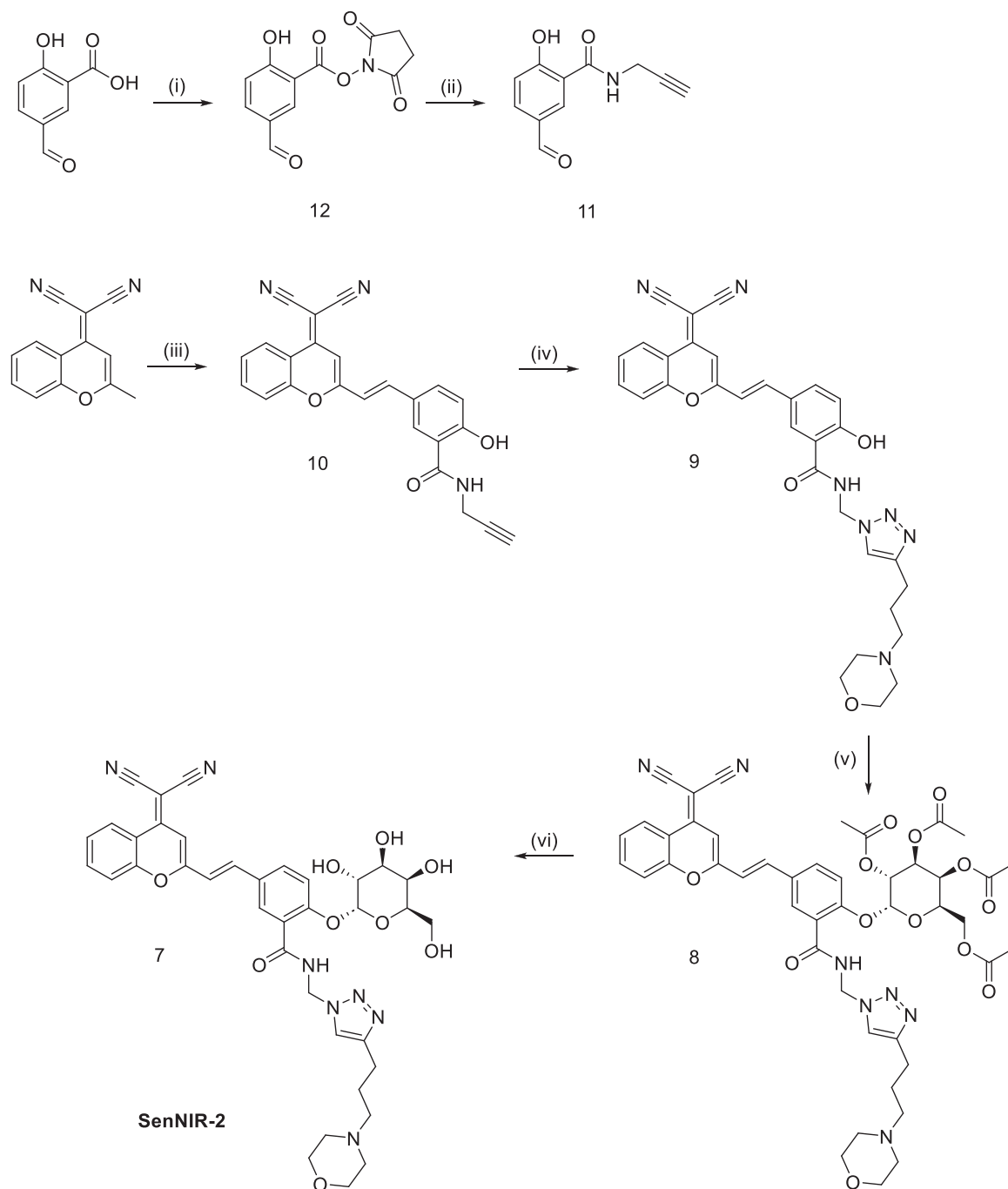
LysoTracker Green DND-26 (Cell Signaling) prior to fixation.

2.11. Ex vivo Imaging

All animal experiments were carried out in accordance with institutional guidelines and were approved by Protocol Approval Committee of the Department of Biology. 5-month-old animals were used as young mature-adult controls, an age at which the senescent-cell burden remains low relative to 24-month-old mice [69]. Mice were sacrificed, tissues were isolated and then washed with PBS three times before being transferred to liquid nitrogen. A total of 5 male C57BL/6 J mice were analyzed per age group. For each organ, 6 independent tissue sections per animal were examined. The frozen tissues were sliced at -20 °C using a cryo-cut microtome. Samples were incubated with 20 μ M of compounds for 1 h at room temperature (RT, 25 °C). For γ H2A.X, samples were fixed with 4% formaldehyde in PBS and permeabilised with 0.2% Triton X-100. After blocking, tissues were sequentially incubated with primary and secondary antibodies for 1 h in RT.

2.12. Computational details

Conformational analysis of the minimum structures of the conjugate of **SenNIR-2** bearing the SA- β -gal trigger group, will be referred to as SA- β -gal-SenNIR-2, and the **SenNIR-1**, **SenNIR-2** and **SenNIR-3** (post-cleavage) have been carried out via semiempirical calculations at the PM6 level of theory. Then the geometry of up to three lowest in energy minimum structures were optimized at the density functional theory (DFT) level (B3LYP [70] and PBE0 [71]) using the 6-31 G(d,p)



Scheme 4. Synthetic route for compound **SenNIR-2** where all intermediates are depicted. The details of the synthesis are pinpointed in the sections below. Reagents and solvents: i) NHS, DCC, dry THF, 25 °C, 30 min, a= 75.7%, ii) TEA, propargylamine, dry ACN, 25 °C, 16 h, a= 88%, iii) compound 11, piperidine (cat.), dry ACN, refluxed, 14 h, a= 56.8%, (iv) compound 5, CuSO₄·5H₂O, sodium ascorbate, DMF, 16 h, 25 °C, a= 50.9%, (v) tetra-O-acetyl-α-D-galactopyranosyl-1-bromide, TEA, 25 °C, ACN 15 h, a= 79.6%, (vi) MeONa, MeOH, 25 °C, 6 h, a= 14.3%.

and 6-311 + G(d,p) [72] basis sets. All calculations were carried out in water solutions employing the polarizable continuum model (PCM) [73]. Frequencies have been calculated for all structures; no imaginary frequencies were found showing that all calculated isomers are true minimum structures. The UV-vis absorption and emission spectra of the studied structures were calculated via the time-dependent DFT (TD-DFT) at the PBE0/6-31 G(d,p) methodology. For the absorption spectrum the 20 lowest in energy excited singlet-spin electronic states were calculated. For the emission spectra the geometries of the S₁ and S₂ excited states were obtained. Additionally, the linear response correction (cLR) approach has been employed for the first two absorption and

emission peaks [74], while the vibronic spectrum of the **SenNIR-2** was calculated using Duschinsky transformation. Finally, ¹H NMR spectra were calculated with respect to the TMS at the B3LYP/6-311 + G(2d,p) level of theory. NMR shielding tensors have been computed with the gauge-independent atomic orbital (GIAO) method. All DFT calculations were carried out with the Gaussian 16 software package [75].

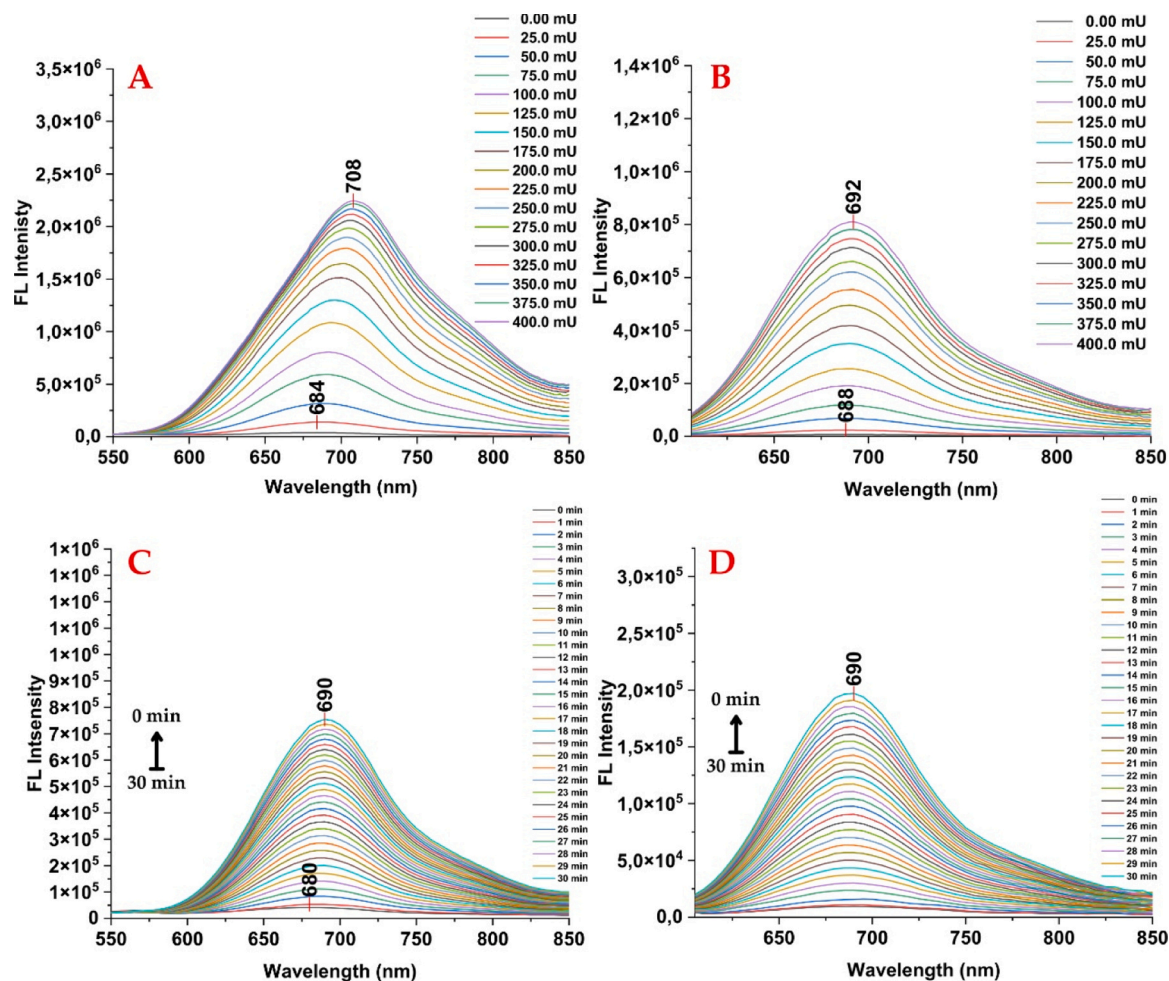


Fig. 6. Spectral properties of SenNIR-2 (10 μ M) in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) (A), (B) Fluorescence spectra of SenNIR-2 upon excitation at 475 and 585 nm, respectively in presence of different concentrations of β -gal. (C),(D) Time dependence of emission spectra (0–30 min) of SenNIR-2 in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 475 and 585 nm, respectively, after the treatment with 0.1 U of β -gal.

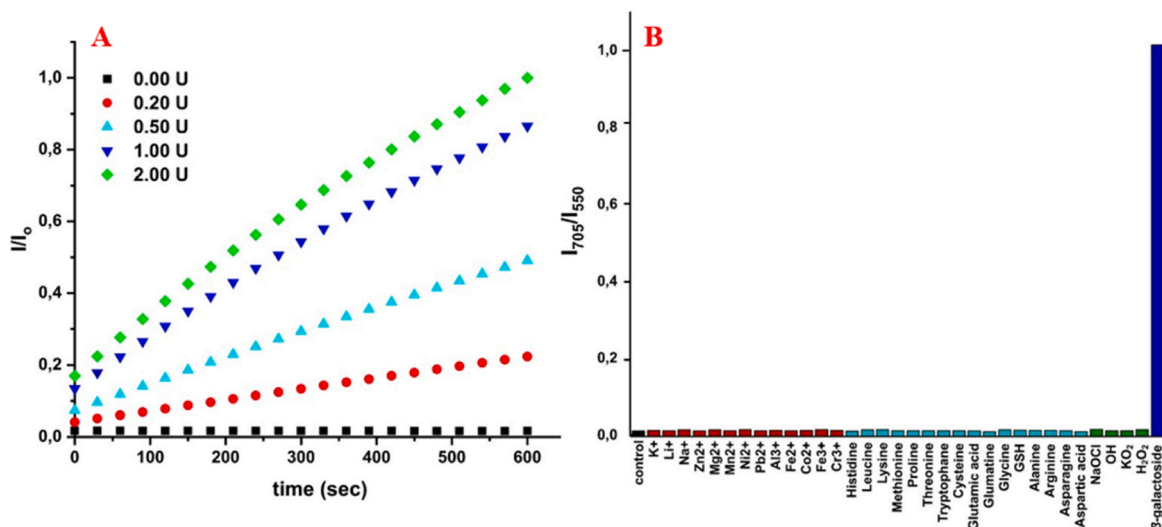


Fig. 7. (A) Kinetics of the fluorescence signal at 690 nm of SenNIR-2 (10 μ M) in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 585 nm in absence and presence of different concentration of β -gal. (B) Fluorescence ratio (I_{705}/I_{550}) responses of SenNIR-2 (10 μ M) to various analytes in DMSO/PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 475 nm.

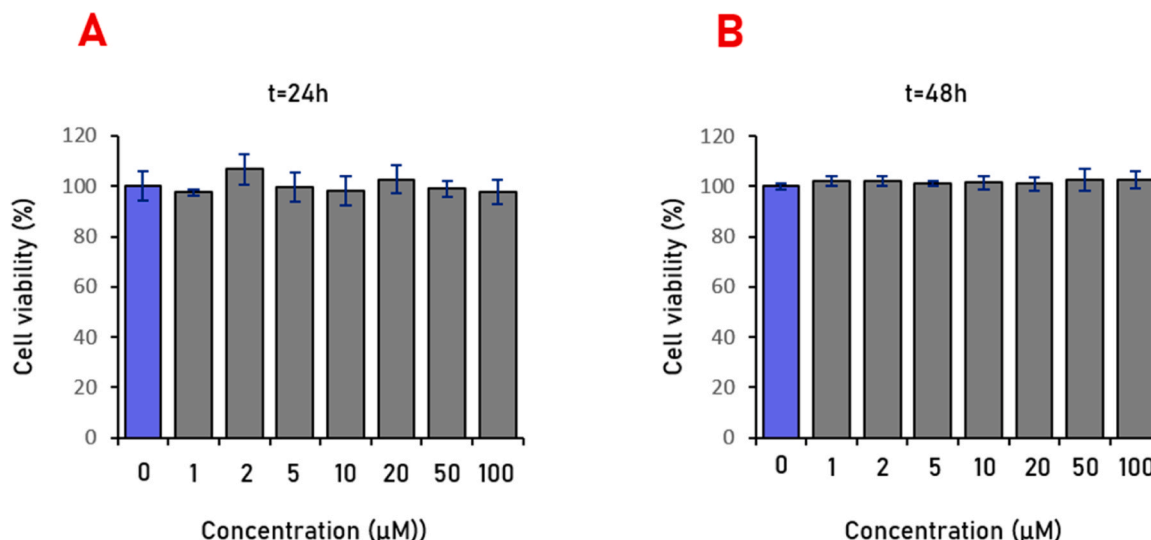


Fig. 8. SenNIR-2 does not exert any cytotoxic effect in human normal skin fibroblasts. Relative (%) survival levels (MTT assay) of BJ skin fibroblasts incubated with the indicated concentrations of the SenNIR-2 for 24 (A) and 48 (B) h. Control samples values (untreated cells) were set to 100%; Bars, $\pm$ SD ($n \geq 2$).

3. Results and discussion

3.1. Rational design for the development of SenNIR

The design of SenNIR was strategically focused on DCM core as a well-established fluorophore known for its enhanced ICT and near-infrared emission. Two orthogonal reactive handles were introduced to this core, enabling simultaneous incorporation of targeting moieties and responsive triggers for selective cellular accumulation and stimulus-activated signal development. To provide chemical flexibility and modularity, an alkyne functional group was incorporated, allowing post-synthetic modification via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). This position was functionalized with an azide-bearing morpholine derivative, ensuring selective lysosomal localization as a key feature for targeting senescent cells. The lysosome was chosen as the targeting destination because SA- β -gal is itself a lysosomal hydrolase; co-localizing the probe with its activating enzyme raises the effective probe concentration at the site of turnover, suppresses off-organelle background, and exploits the acidic lysosomal pH at which SA- β -gal remains selectively active in senescent cells, together improving the sensitivity and spatial specificity of the turn-on response. In this structural assembly, the triazole ring plays a vital structural role. It serves as a robust and metabolically stable linker connecting the lysosomal-targeting morpholine unit to the fluorophore core, maintaining the necessary molecular architecture without disrupting the primary D- π -A electronic pathway. To enable responsiveness to SA- β -gal, the second reactive handle was coupled to a β -gal-cleavable trigger group, designed to suppress fluorescence through disruption of the ICT process. In the inactive state, the galactosyl trigger diminishes the electron-donating ability of the phenolic oxygen, suppressing the ICT and keeping the probe quenched. Upon enzymatic cleavage by SA- β -gal the strongly electron-donating hydroxyl group restores the ICT process, driving electron density towards the DCM acceptor and producing a strong NIR fluorescence response. This mechanism could enable non-invasive, ratiometric optical imaging of lysosomal SA- β -gal activity, offering both spatial resolution and biochemical specificity (Scheme 1). This rational design could afford a modular platform with potential applications in senescence-targeted therapeutic strategies.

3.2. Synthetic route of SenNIR-1

SenNIR-1, was synthesized via a multi-step sequence involving alkylation, azidation, and a CuAAC "click" reaction, followed by

glycosylation. Briefly, morpholine was alkylated with 3-iodo-1-chloropropane in refluxing toluene, and the resulting intermediate was converted to the corresponding azide using sodium azide (NaN_3) in a H_2O /methanol (MeOH) mixture (Scheme 2).

The core fluorophore was then assembled through a click reaction between the azido-intermediate and compound 4 in the presence of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ and sodium ascorbate in dimethylformamide (DMF). Subsequent glycosylation with tetra-O-acetyl- α -D-galactopyranosyl-1-bromide, followed by final deprotection using sodium methoxide (MeONa) in dry MeOH, afforded SenNIR-1 as a brown solid. All intermediates were purified and characterized by ^1H NMR, ^{13}C NMR. The final product was further characterized by mass spectrometry, and its purity was confirmed by analytical HPLC (Figure S1-S12).

3.3. Photophysical properties and selectivity of SenNIR-1

To monitor the responsiveness of SenNIR-1 towards β -gal UV-Vis and fluorescence studies were performed. As illustrated in Figure S13-15, SenNIR-1 in DMSO: phosphate buffer (PB, 3:7, 10 mM pH 6.00) displays a distinct absorption spectrum with λ_{max} centered at 425 nm. Upon the gradual addition of β -gal (up to 200 mU), a distinct 160 nm bathochromic shifting (red shift) was observed, accompanied by the presence of one isosbestic point centered at 465 nm. This finding could be explained by the hydrolysis of the sugar moiety attached to the phenolic group of compound 3 resulting in its transformation into compound 1.

Furthermore, we evaluated the fluorescent responsiveness of SenNIR-1 in the presence of β -gal upon excitation at 465 and 585 nm. With the gradual addition of β -gal (0-200 mU) upon excitation at the isosbestic point of the absorbance spectra the ratiometric fluorescent behavior (I_{690}/I_{500}) of the probe was recorded. As illustrated in Fig. 1A, the enhancement of the fluorescent signal was significant, accompanied by a bathochromic shifting into the emission range of 550-850 nm along with a decrease in the emission band at 500 nm. More specifically, the ratiometric fluorescent signal (I_{690}/I_{500}) was significantly 8-fold increased. Moreover, a distinct 20-fold enhancement at 690 nm was observed upon excitation at 585 nm (Fig. 1B). This finding could be explained by the β -gal mediated hydrolysis of the sugar moiety that releases compound 3 restoring in this way the ICT process. Next, we recorded the fluorescent spectra of SenNIR-1 as a function of time in the presence of 100 mU of the enzyme. As illustrated in Fig. 1C,D, the fluorescent signal gradually increased upon excitation at 465 and 585 nm. The ratiometric fluorescent signal (I_{690}/I_{500}) as well as the

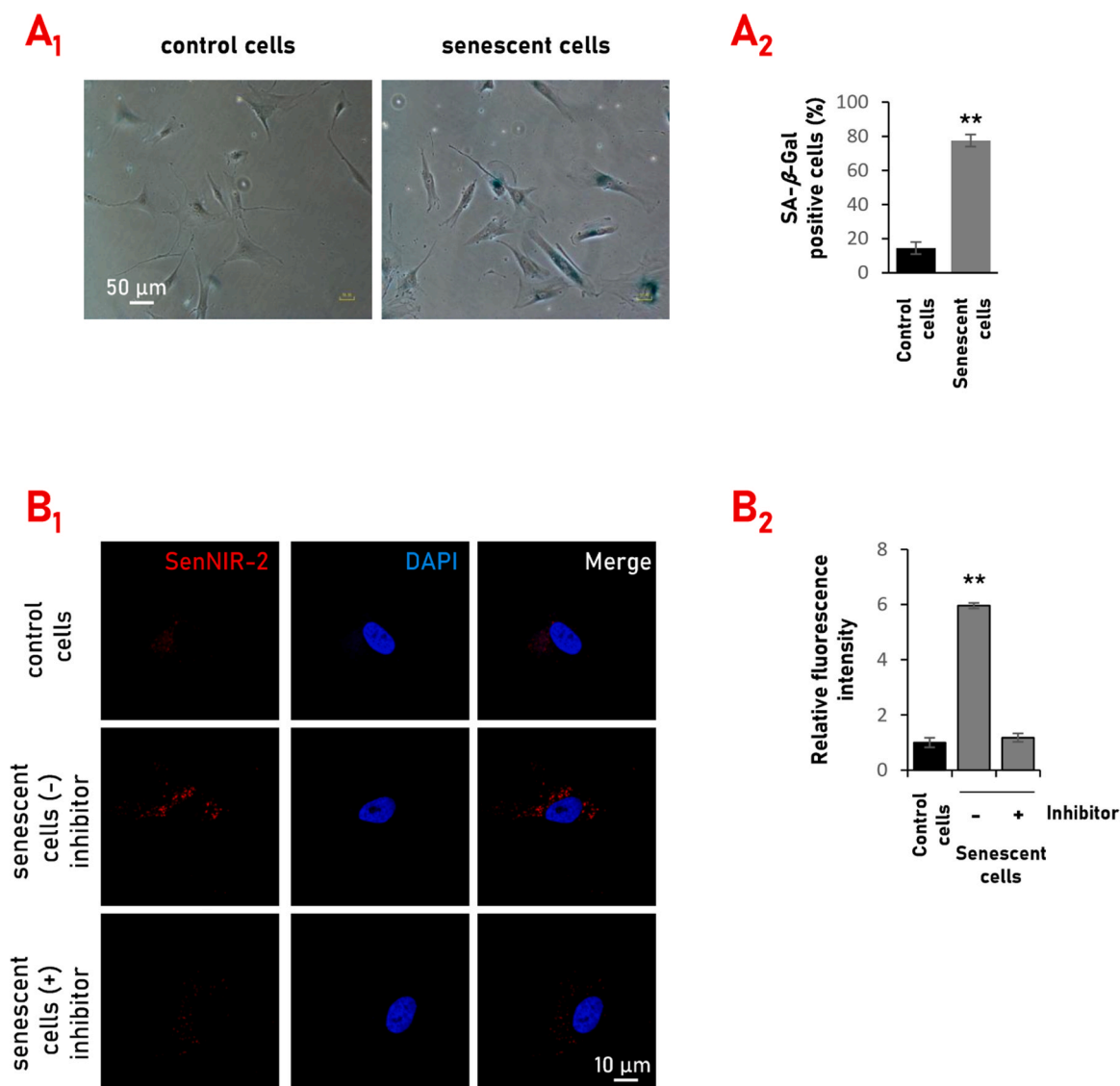


Fig. 9. SenNIR-2 visualizes SA-β-gal activity in senescent cells. (**A₁**) Representative light field images following SA-β-gal staining of control BJ cells or cells treated (three exposures of 48 h each) with 300 μM H₂O₂. (**A₂**) Relative (%) number of SA-β-gal positive cells following treatment of cells exactly as in (**A₁**). CLSM visualization (**B₁**) and quantitation of SenNIR-2 fluorescence intensity (**B₂**) in control and premature senescent (H₂O₂ treated) cells. Cells were cultured on coverslips and incubated with 10 μM of SenNIR-2 for 1 h, upon or without pretreatment with the β-gal inhibitor (D-galactose; 1 mM) for 1 h. Cells were then fixed, stained with DAPI and mounted on slides before observation at CLSM following excitation at 543 nm. Control samples values (untreated cells) were set to 1 (**B₂**). Bars ± SD. ** $p < 0.01$. Bars, in (**A₁**) 50 μm and in (**B₁**) 10 μm.

fluorescent signal at I₆₉₀ displayed good linear relationship in this set of experiments.

To evaluate the response rate of SenNIR-1 in the presence of β-gal we conducted fluorescent time-course experiments by monitoring the fluorescence signal at 690 nm in the presence of different concentrations of the enzyme. As illustrated in Fig. 2A in the presence of 0.5 U and 1.0 U of the enzyme, the fluorescent signal of the probe reaches its maximum value within 240 s and 480 s, respectively. The detection limit was calculated at 0.49 mU, indicating that SenNIR-1 has high sensitivity to changes of β-gal concentrations. The selectivity of this probe towards different analytes including metals, aminoacids and ROS was further evaluated via fluorescence spectroscopy by monitoring the ratiometric fluorescent signal (I₆₉₀/I₅₀₀). As illustrated in Fig. 2B, the ratiometric fluorescent signal was barely altered by the addition of any analytes (100 μM) [except β-gal (200 mU)], indicating the enhanced selectivity of SenNIR-1.

3.4. Cytotoxicity evaluation of SenNIR-1

Then, we investigated the effect of SenNIR-1 on the viability of BJ skin fibroblasts by performing the MTT toxicity assay. For this purpose, cells were incubated with increasing concentrations of SenNIR-1 for 24 and 48 h (Fig. 3). We found that the probe did not exert any cytotoxic effect in human normal fibroblasts.

3.5. Bioimaging of endogenous SA-β-gal in cells

We then examined whether SenNIR-1 can be used to track and visualize β-gal activity in senescent cells. Thus, we triggered SIPS by exposing young proliferating BJ fibroblasts to the oxidative agent H₂O₂. H₂O₂ is the most widely used agent to induce SIPS in normal cells, leading to the overexpression of SA-β-gal [76]. Herein, we performed three exposures (48 h each) of cells to 300 μM H₂O₂ and, as shown in Fig. 4A, 80 ± 7% of cells were stained positive in SA-β-gal staining post-H₂O₂ treatment. Normal untreated BJ cells were used as control group.

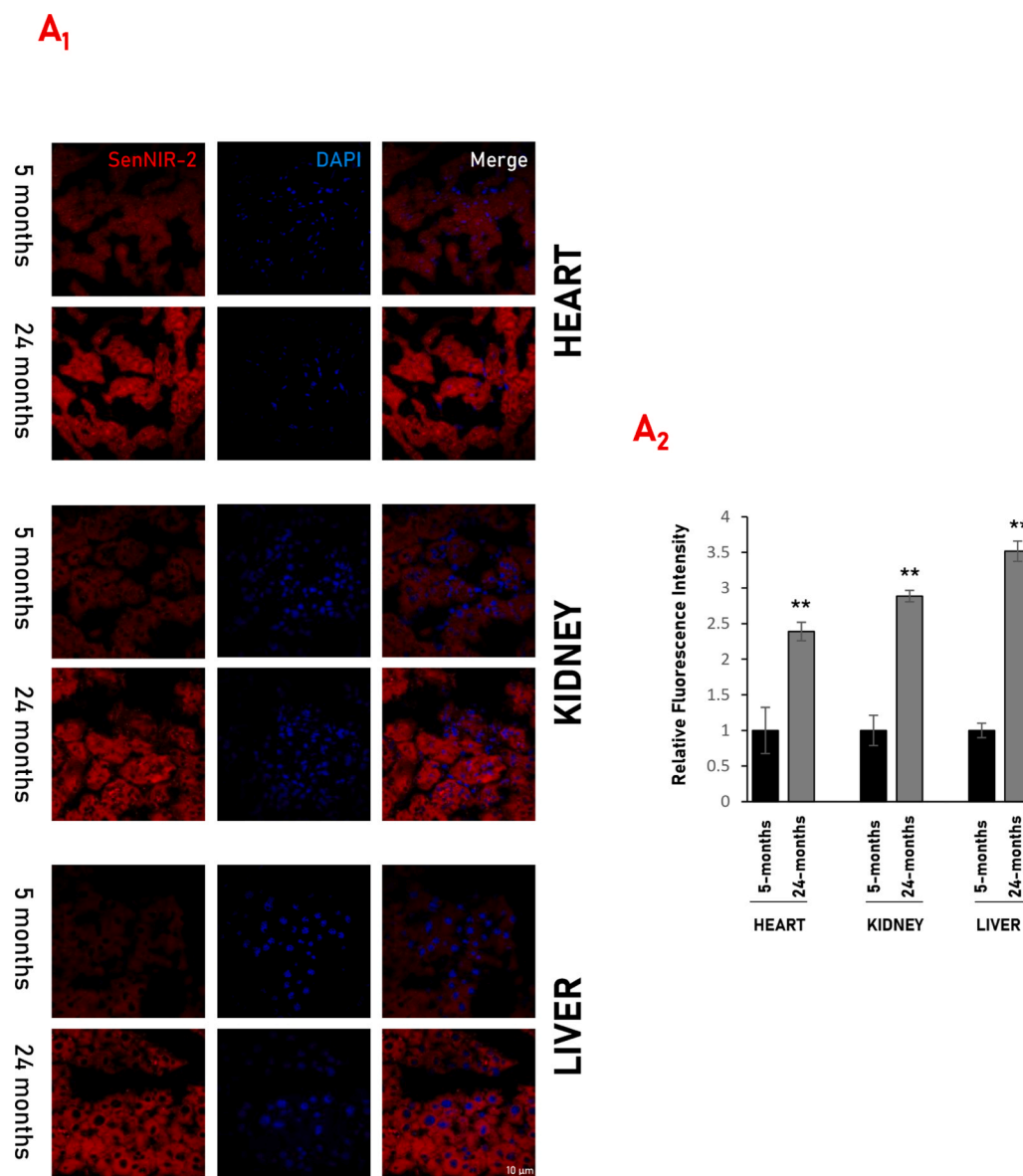


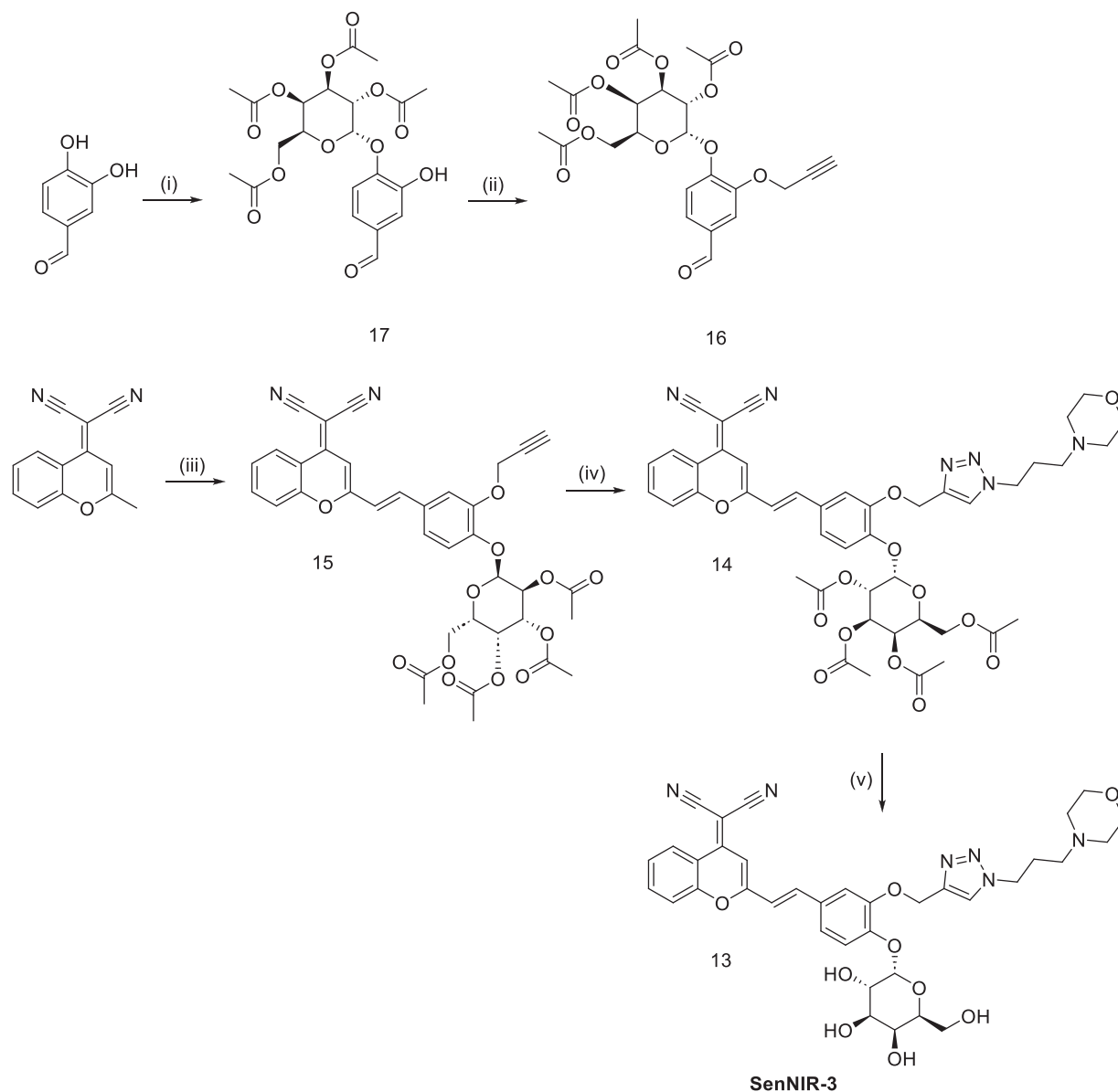
Fig. 10. (A₁) Representative images of heart, kidney, and liver sections from 5- and 24-month-old C57BL/6 J male mice incubated with **SenNIR-2** (20 μ M). Scale bar: 10 μ m. (A₂) Relative (%) fluorescence of **SenNIR-2** (20 μ M) in tissue sections from 5-, and 24-month-old C57BL/6 J male mice. Control (5months old) samples values were set to 1. Bars, $\pm$ SD; * $p < 0.05$; ** $p < 0.01$. $n = 5$ mice per age group; 6 sections per organ per animal.

Both control and H₂O₂-treated BJ cells were incubated with **SenNIR-1** (10 μ M) in culture medium for 1 h at 37 $^{\circ}$ C and were then observed with a Digital Eclipse C1 Nikon CLSM upon excitation of the probe at 543 nm. As shown in Fig. 4B, strong fluorescence was observed in senescent cells, whereas only minimal fluorescence was detected in young replicating (control) cells. As a negative control, both control and senescent cells were pretreated with the β -gal competitive inhibitor, namely D-galactose (1 mM) for 1 h and were then incubated with the **SenNIR-1** (10 μ M) for 1 h at 37 $^{\circ}$ C. Notably, the senescence-associated increased fluorescence signal produced in senescent cells by **SenNIR-1** was barely observed after preincubation with D-galactose (Fig. 4B), further supporting the capability of the probe to sense β -gal activity specifically in senescent cells. To verify lysosomal localization, cells were co-stained with LysoTracker and **SenNIR-1**. A strong overlap between the fluorescence signals was observed, confirming that **SenNIR-1** preferentially accumulates within lysosomal compartments and validating the effectiveness of the morpholine-based targeting unit (Fig. 4C). To further test whether this accumulation depends on the acidic lysosomal environment, parallel cells were pre-treated with chloroquine (CQ) 100 μ M, a

lysosomotropic agent that raises lysosomal pH. CQ pre-treatment markedly attenuated the punctate lysosomal NIR signal of **SenNIR-1** (Fig. 4D).

3.6. Ex vivo bioimaging evaluation of **SenNIR-1**

Given the fact that **SenNIR-1** selectively visualized SA- β -gal activity in senescent cells, we then investigated its activity *ex vivo*. To this point, three main organs, namely heart, liver, and kidney from 5- and 24-month-old C57BL/6 J male mice, were isolated and sliced (see Materials and Methods). The tissue sections were treated with **SenNIR-1** for 1 h and observed by CLSM. As illustrated in Fig. 5, we found that fluorescence intensity was increased significantly in all tissues from 24-months old mice, with the fluorescence levels in aged heart and kidneys being six times higher than those seen in these organs when derived from young mice. To confirm our results, we stained our samples with a marker of DNA damage and genotoxic stress, namely γ H₂A.X⁴⁸ [77], as expected more DNA damage foci were noted in cells nuclei from aged tissues.



Scheme 5. Synthetic route for compound **SenNIR-3** where all intermediates are depicted. The details of the synthesis are pinpointed in the sections below. Reagents and solvents: **i)** tetra-O-acetyl- α -D-galactopyranosyl-1-bromide, K_2CO_3 , dry ACN, 25 °C, 16 h, $a = 71.2\%$, **ii)** propargyl bromide, K_2CO_3 , dry ACN, 25 °C, 16 h, $a = 98.7\%$, **iii)** compound **16**, piperidine (cat.), dry ACN, refluxed, 14 h, $a = 32\%$, **iv)** compound **5**, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF, 25 °C, 16 h, $a = 20.5\%$, **v)** MeONa, MeOH, 25 °C, 6 h, $a = 34\%$.

3.7. Impact of molecular architecture on *SenNIR* probe performance via structural analogues

To elucidate the influence of molecular design on **SenNIR** performance, we synthesized a series of structural analogues based on the lead candidate **SenNIR-1**. These analogues were strategically engineered to evaluate how variations in the spatial arrangement and connectivity of key functional units affect the photophysical and biological properties of the probe. Specifically, we employed different molecular architectures to tether the three core elements of the **SenNIR** scaffold: (i) the SA- β -gal responsive trigger, responsible for selective activation; (ii) the morpholine moiety, which directs the probe to lysosomes; and (iii) the dicyanomethylene-4H-pyran fluorophore, which provides the NIR fluorescent signal.

By systematically modulating the spatial arrangement and chemical nature of the linkage between these domains, we aimed to assess the impact of molecular geometry on SA- β -gal responsiveness, lysosomal

targeting efficiency, cytocompatibility, and NIR fluorescence output in vitro and ex vivo (Scheme 3).

The structural modifications focused specifically on the regiochemical substitution pattern of the lysosomal-targeting unit relative to the phenolic oxygen atom of the enzymatic trigger. In the lead architecture, **SenNIR-1**, the morpholine group is tethered via an ether bond at the *meta* position with respect to the glycosidic linkage of the trigger. In contrast, **SenNIR-2** introduces two concurrent modifications, the substitution site of the targeting moiety is relocated to the *ortho* position relative to the sugar trigger, and the linkage is converted to an amide bond. This shift significantly alters the electronic and steric environment surrounding the fluorophore core. **SenNIR-3** represents a hybrid design that retains the *ortho* regiochemistry (relative to the phenolic oxygen) observed in **SenNIR-2** while preserving the ether linkage chemistry utilized in **SenNIR-1**.

These rational structural variations were designed to systematically explore the influence of positional isomerism and linker chemistry on

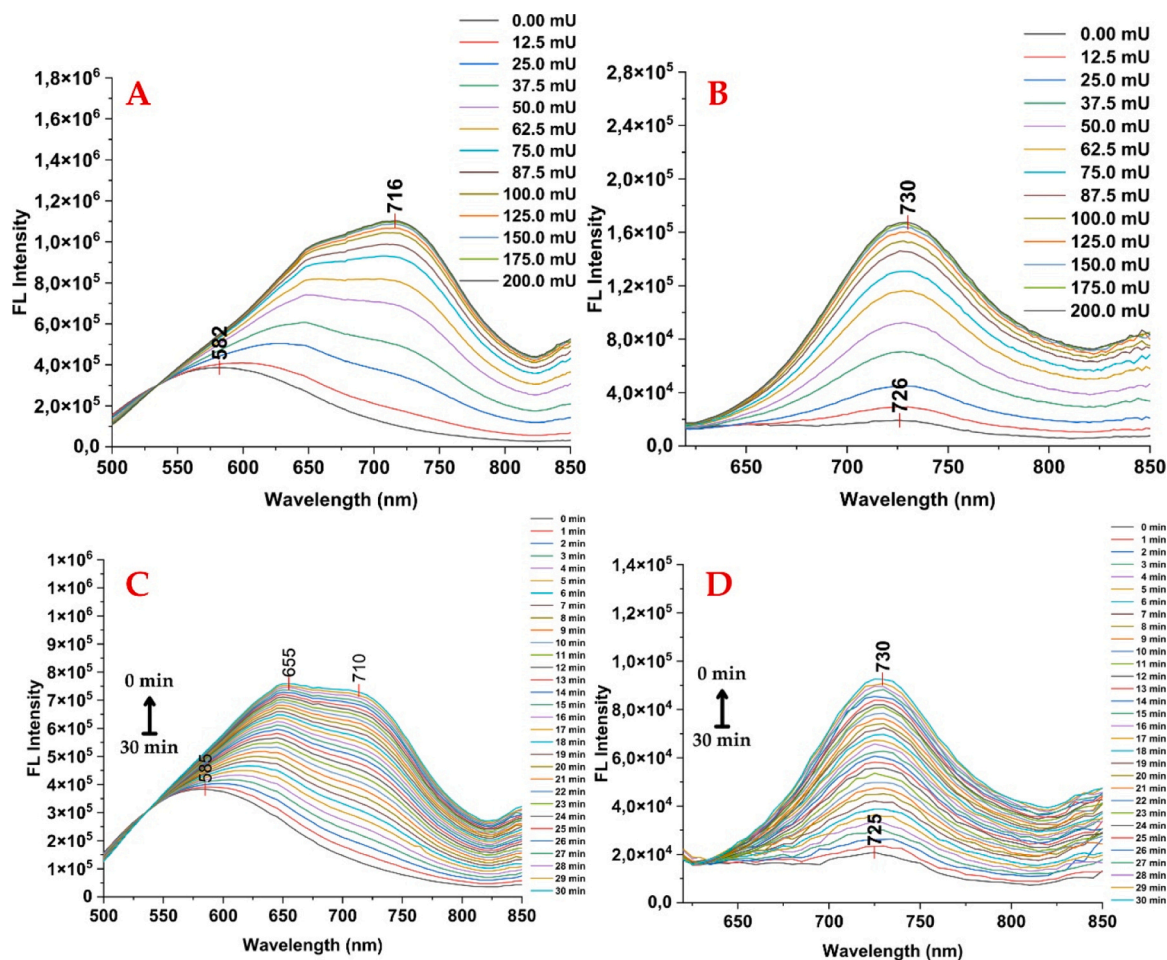


Fig. 11. Spectral properties of **SenNIR-3** (10 μ M) in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) (A), (B) Fluorescence spectra of **SenNIR-3** upon excitation at 465 and 585 nm, respectively in presence of different concentrations of β -gal. (C), (D) Time dependence of emission spectra (0–30 min) of **SenNIR-3** in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 465 and 585 nm, respectively, after the treatment with 0.15 U of β -gal.

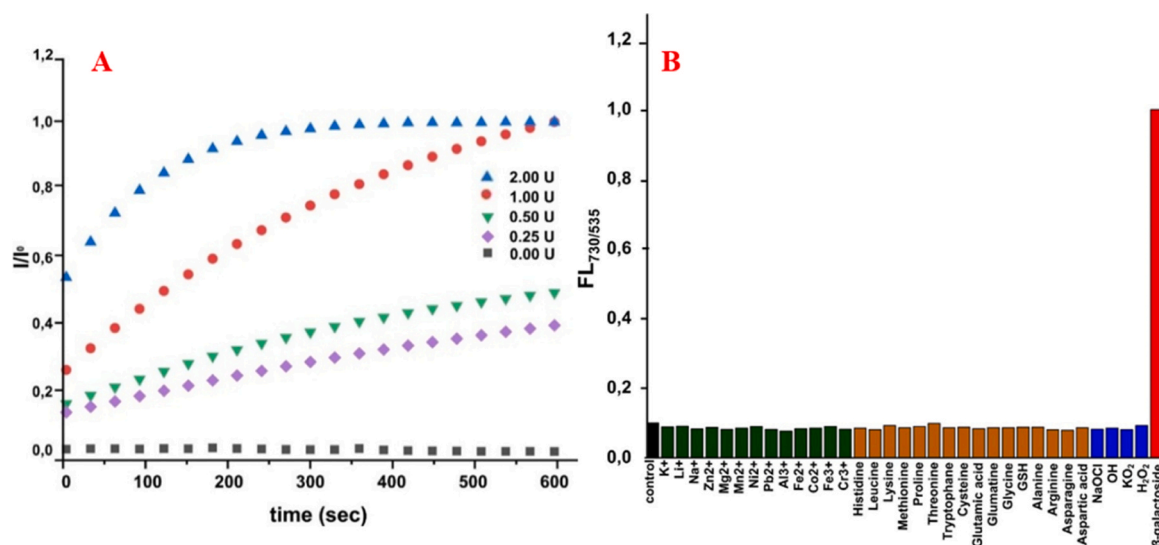


Fig. 12. (A) Kinetics of the fluorescence signal at 730 nm of **SenNIR-3** (10 μ M) in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 585 nm in absence and presence of different concentration of β -gal. (B) Fluorescence ratio (I_{705}/I_{550}) responses of **SenNIR-3** (10 μ M) to various analytes in DMSO: PB (3:7 v/v, 10 mM, pH 6.00) upon excitation at 475 nm.

the probe's overall behavior, particularly its enzymatic responsiveness to β -gal. This structure-based investigation could provide valuable

insight into the design principles governing the performance and optimization of the **SenNIR** probe platform.

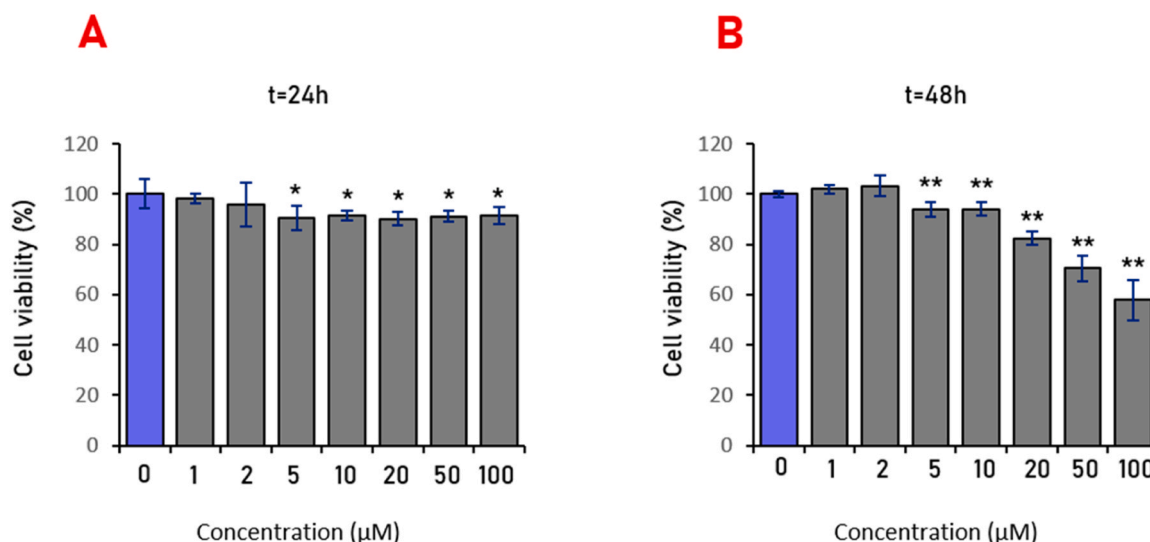


Fig. 13. **SenNIR-3** displays mild cytotoxic effects at higher concentration in human normal skin fibroblasts. Relative (%) survival levels (MTT assay) of BJ skin fibroblasts incubated with the indicated concentrations of the **SenNIR-3** for 24 (A) or 48 (B) h. Control samples values (untreated cells) were set to 100%. Bars, $\pm$ SD; * $p < 0.05$; ** $p < 0.01$.

3.8. Synthetic route of **SenNIR-2**

The synthesis of the structural analogues followed a systematic multi-step protocol. Briefly, 5-formylsalicylic acid was activated using N-hydroxysuccinimide (NHS) and N,N'-dicyclohexylcarbodiimide (DCC) in anhydrous tetrahydrofuran (THF) to yield the corresponding ester. Subsequent amidation with propargylamine in the presence of triethylamine (TEA) afforded the alkyne-functionalized intermediate 11 (Scheme 4).

The NIR fluorophore core was constructed via a Knoevenagel condensation between compound 11 and 2-(2-methyl-4H-chromen-4-ylidene) malononitrile, catalyzed by piperidine in refluxing acetonitrile. The resulting alkyne-bearing fluorophore (10) was then coupled with the azido-intermediate 5 via a copper-catalyzed "click" reaction, yielding the precursor 9. The final steps involved glycosylation with tetra-O-acetyl- α -D-galactopyranosyl-1-bromide followed by deprotection of the acetyl groups using MeONa in dry MeOH to afford the target probe as a brown solid. Detailed synthetic procedures, yields, and spectroscopic characterization are provided in the SI (Figures S16–27).

3.9. Spectral properties and selectivity of **SenNIR-2**

SenNIR-2 maintains a core structure similar to **SenNIR-1**, with the primary structural differentiation being the linkage of the targeting moiety; specifically, an amide bond is utilized instead of the ether linkage found in **SenNIR-1**. While the enzymatic target (β -gal) and the molecular trigger remain consistent between the two probes, this structural variation was systematically investigated to evaluate whether the linkage chemistry influences the probe's photophysical properties or enzymatic accessibility.

To evaluate the reactivity of **SenNIR-2** toward β -gal, fluorescence spectroscopy was performed in a DMSO:PB buffer system (3:7, 10 mM, pH 6.00) (Figure S28–30). As illustrated in Fig. 6A, B, **SenNIR-2** was analyzed upon excitation at both 475 nm and 585 nm. The gradual addition of β -gal (0–400 mU) prompted a distinct bathochromic shift in the emission maximum from 690 nm to 705 nm. Specifically, excitation at 475 nm resulted in a 60-fold enhancement in intensity at 690 nm, while excitation at 585 nm yielded a 70-fold enhancement. This significant fluorescence "turn-on" response is attributed to the enzyme-mediated hydrolysis of the sugar moiety, which releases the free fluorophore (compound 9) and subsequently restores the ICT process.

Furthermore, the kinetic profile of **SenNIR-2** was monitored in the presence of 100 mU of the enzyme over time. Following excitation at 475 nm and 585 nm, the fluorescent signal exhibited a gradual increase of approximately 20-fold (Fig. 6C, D). Under these experimental conditions, the fluorescence intensities at I_{705} and at I_{690} demonstrated strong linear relationships, confirming the reliability of the probe for quantitative enzymatic sensing.

Time-course fluorescence studies were conducted by monitoring the emission signal at 690 nm (under 585 nm excitation) to evaluate the response kinetics of **SenNIR-2** across a range of enzyme concentrations. As the concentration of SA- β -gal was increased to 1.0 U and 2.0 U, the fluorescence intensity exhibited a near-linear enhancement, as depicted in Fig. 7A. To further validate the specificity of the probe, the selectivity of **SenNIR-2** was assessed against a library of potential analytes, including metal ions, amino acids, and ROS, by monitoring the ratiometric fluorescent signal I_{705}/I_{550} . As illustrated in Fig. 7B, the addition of these analytes (100 μ M) induced negligible changes in the ratiometric signal. In contrast, the addition of SA- β -gal (400 mU) triggered a robust response, demonstrating the high selectivity and reliability of the probe for detecting senescence-associated enzyme activity in complex environments.

3.10. Cytotoxicity evaluation of **SenNIR-2**

Cytotoxicity of **SenNIR-2** was examined on BJ fibroblasts by performing the MTT assay with increasing concentrations of **SenNIR-2** for 24 and 48 h. As shown in Fig. 8, we found no significant toxicity in human normal fibroblasts.

3.11. Bioimaging of endogenous SA- β -gal in cells

SenNIR-2 was also evaluated for its ability to visualize β -gal specifically in senescent cells. Following the same procedure as for **SenNIR-1**, a model of SIPS induction in cells was created by three continuous exposures of young replicating BJ fibroblasts to H_2O_2 . Normal untreated and H_2O_2 -treated BJ cells were incubated with 10 μ M of **SenNIR-2** in culture medium for 1 h at 37 °C. Cells treated with 1 mM of D-galactose were used also as a negative control. Our analyses showed that senescent (H_2O_2 treated) cells (Fig. 9A) exhibited significantly higher levels of staining intensity (CLSM) after exposure to **SenNIR-2** in comparison with control and cells treated with D-

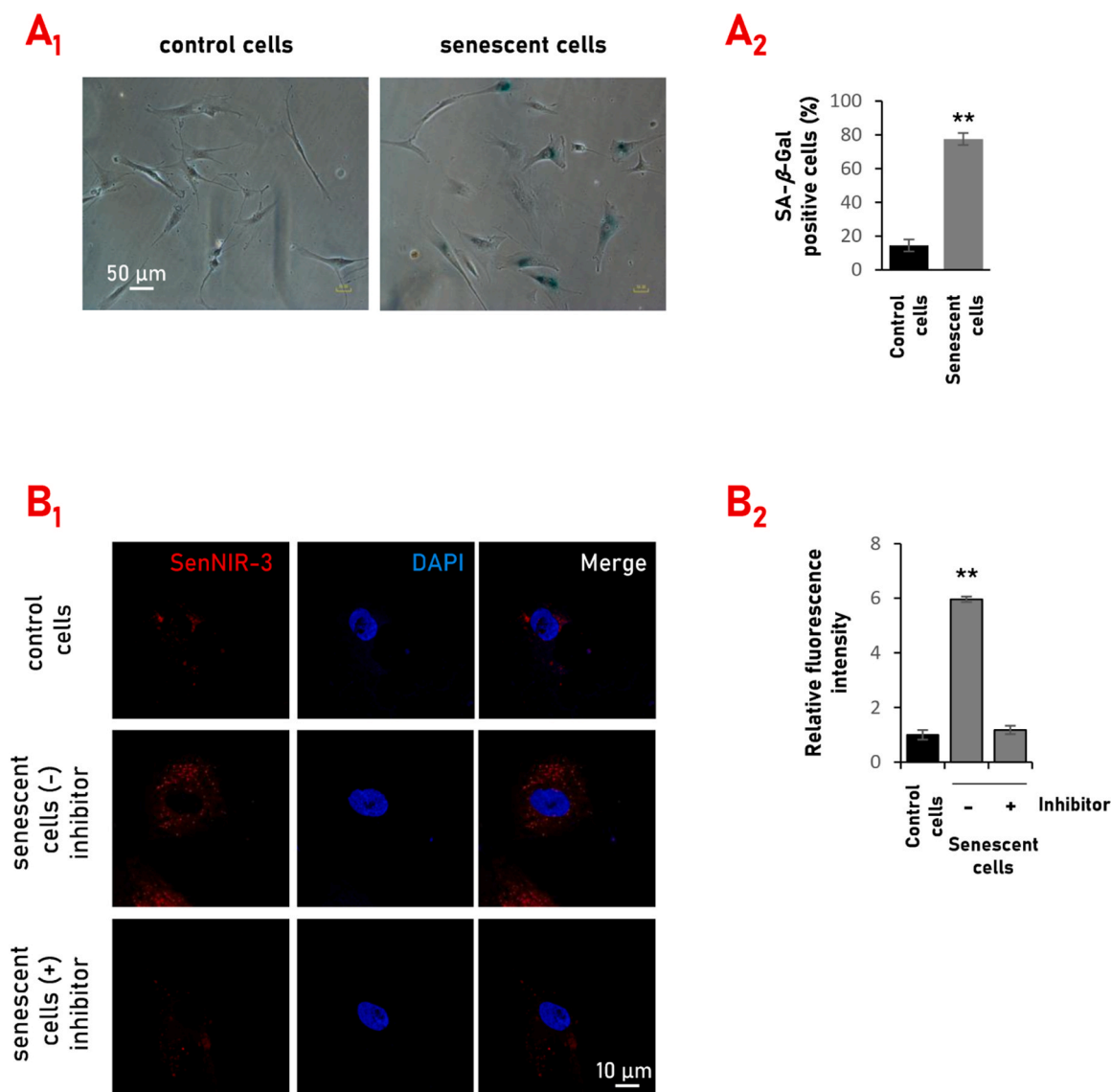


Fig. 14. SenNIR-3 visualizes SA- β -gal activity in senescent cells. (**A₁**) Representative light field images following SA- β -gal staining of control BJ cells or cells treated (three exposures of 48 h each) with 300 μ M H₂O₂. (**A₂**) Relative (%) number of SA- β -gal positive cells following treatment of cells exactly as in (**A₁**). CLSM visualization (**B₁**) and quantitation of intensity (**B₂**) of SenNIR-3 fluorescence in control and premature senescent (H₂O₂ treated) cells. Cells were cultured on coverslips and incubated with 5 μ M of SenNIR-3 for 1 h, upon or without pretreatment with the β -gal inhibitor (D-galactose; 1 mM) for 1 h. Cells were then fixed, stained with DAPI and mounted on slides before observation under a CLSM following excitation at 543 nm. Bars $\pm$ SD. ** $p < 0.01$. Control samples values (untreated cells) were set to 1 (**B₂**). Bars, in (**A₁**) 50 μ m and in (**B₁**) 10 μ m.

galactose (Fig. 9B).

3.12. Ex vivo bioimaging evaluation of SenNIR-2

Given our findings, we further tested SenNIR-2 for its activity *ex vivo*. As mentioned before sections from three main organs, namely heart, liver, and kidney from 5- and 24-month-old C57BL/6 J male mice, were treated with SenNIR-2 for 1 h and evaluated by CLSM. Our findings in cells were verified also in tissues since older sliced tissues illustrated more intense signal than younger controls. (Fig. 10).

3.13. Synthetic route of SenNIR-3

Compounds SenNIR-1 and SenNIR-3 are structurally very analogous. Both scaffolds utilize an etheric linkage to tether the lysosomal targeting moiety; however, the key distinction lies in the substitution pattern on the central phenyl ring. Specifically, whereas the lead

compound SenNIR-1 features a *meta*-substitution pattern, SenNIR-3 adopts an *ortho*-substitution pattern relative to the phenolic oxygen atom where the galactosyl trigger is attached Scheme 5.

The synthesis of SenNIR-3 was initiated by the selective glycosylation of 3,4-dihydroxybenzaldehyde with tetra-O-acetyl- α -D-galactopyranosyl-1-bromide in the presence of K₂CO₃, yielding intermediate 17. This was followed by substitution of the phenolic group using propargyl bromide to afford the alkyne-functionalized precursor 16.

The NIR-emitting core was then constructed via a piperidine-catalyzed Knoevenagel condensation between compound 16 and 2-(2-methyl-4H-chromen-4-ylidene) malononitrile in refluxing acetonitrile. The resulting intermediate (15) underwent a Cu-catalyzed click reaction with the azido-compound 5 to yield the protected precursor 14. Final deprotection of the galactosyl moiety was performed using MeONa in anhydrous MeOH, and the target probe was isolated as a brown solid. Full experimental details, including reaction conditions and

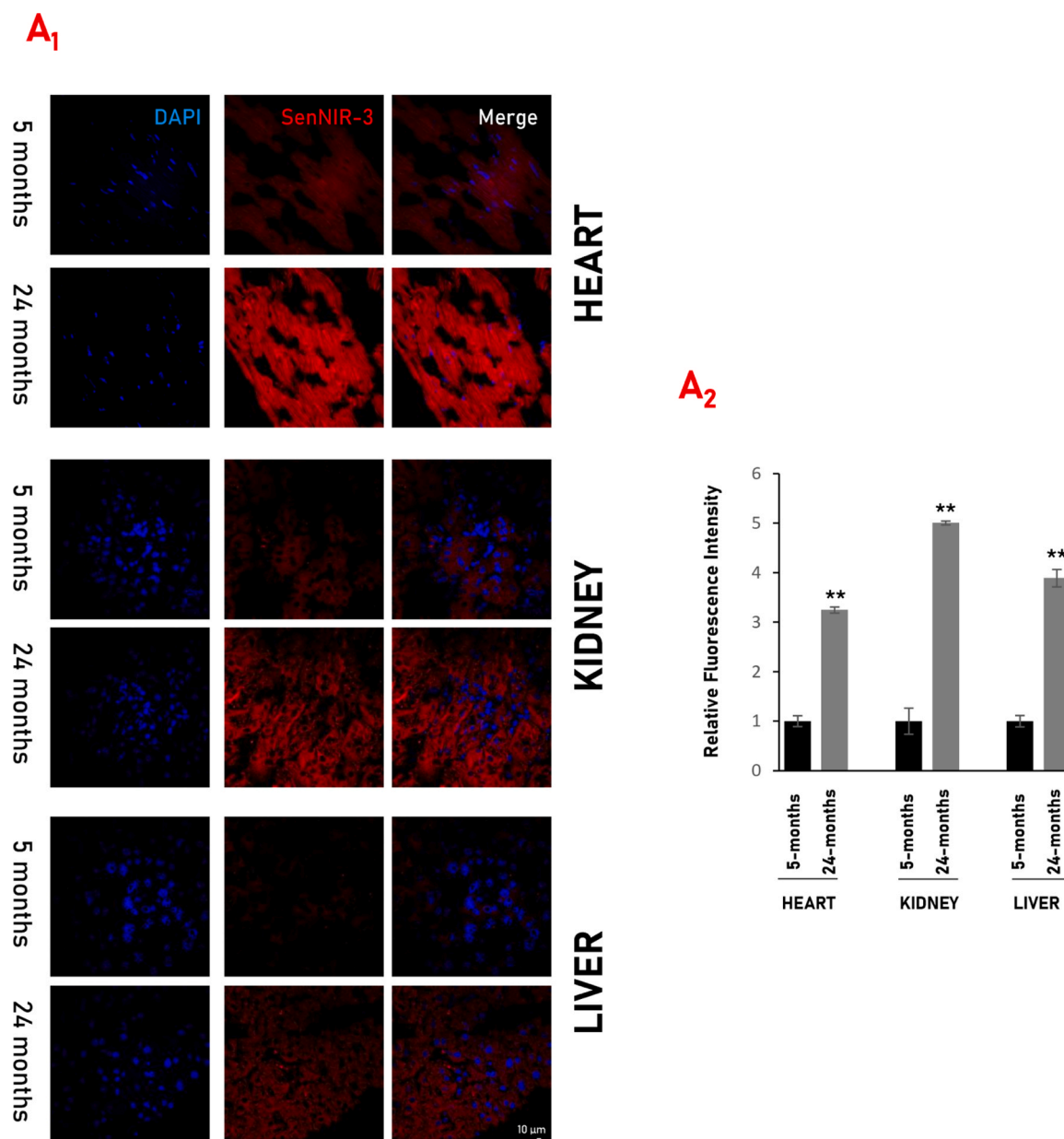


Fig. 15. (A₁) Representative images of heart, kidney, and liver sections from 5- and 24-month-old C57BL/6 J male mice following incubation with **SenNIR-3** (20 μ M). Scale bar: 10 μ m. (A₂) Relative (%) fluorescence of **SenNIR-3** (20 μ M) in tissue sections from 5-, and 24-month-old C57BL/6 J male mice. Control (5months old) samples values were set to 1. Bars, $\pm$ SD; * $p < 0.05$; ** $p < 0.01$. $n = 5$ mice per age group; 6 sections per organ per animal.

Table 2

Photophysical Properties and Responsiveness of **SenNIR** Probes.

Compound	λ_{exc} (nm)	λ_{em} (nm)	Responsiveness (with β -gal)
SenNIR-1	465	575	37 nm redshift (to 612 nm) + 3-fold increase
SenNIR-1	585	688	~17-fold increase at 688 nm + shoulder ~770 nm
SenNIR-2	475	685	23 nm redshift (to 708 nm) + 54-fold increase
SenNIR-2	585	690	~119-fold increase at 690 nm + shoulder ~775 nm
SenNIR-3	465	582	134 nm redshift (to 716 nm) + 8-fold increase at 700 nm
SenNIR-3	585	730	~9-fold increase at 728 nm

spectroscopic characterization are provided in the SI (Figures S31–42).

3.14. Spectral properties and senescence selectivity of **SenNIR-3**

To evaluate the enzymatic responsiveness of **SenNIR-3** toward β -gal, fluorescence titration experiments were performed in a DMSO:PB buffer system (3:7 v/v, 10 mM, pH 6.00) as illustrated at Figure S43–45. Following the protocol established for the previous analogues, the probe was analyzed upon excitation at both 465 nm and 585 nm. As illustrated in Fig. 11A,B, the gradual addition of the enzyme (0–400 mU) led to a significant bathochromic shift in the emission maximum from 572 nm to 730 nm. Specifically, excitation at 465 nm resulted in a distinct 12-fold enhancement in fluorescence intensity at the 730 nm peak, while excitation at 585 nm yielded a 9-fold increase at the same wavelength. This "turn-on" response is attributed to the enzyme-mediated cleavage of the galactosyl moiety, which restores the electron-donating capability of the phenolic group and re-establishes the ICT process. The shift into the deep NIR region (730 nm) for **SenNIR-3** highlights the critical impact of its *ortho*-substitution pattern on the

Table 3

Quantitative photophysical parameters and turn-on senescence sensing performance of the SenNIR probe series.

Compound	Relative Quantum Yield ^a	Absolute Quantum Yield (Φ) ^a	Enhancement Factor (Turn-On)	ϵ (M ⁻¹ cm ⁻¹)	Brightness ($\epsilon \cdot \Phi$)
SenNIR-1	0.074	0.0008 (0.08%)		29,800 $\pm$ 253	23.8
SenNIR-1 after cleavage	0.323	0.0036 (0.36%)	~4.3-fold increase	17,300 $\pm$ 219	62.3
SenNIR-2	0.074	0.0008 (0.08%)		29,170 $\pm$ 386	23.3
SenNIR-2 after cleavage	0.424	0.0047 (0.47%)	~5.7-fold increase	18,710 $\pm$ 177	87.9
SenNIR-3	0.219	0.0024 (0.24%)		30,190 $\pm$ 252	72.5
SenNIR-3 after cleavage	0.462	0.0051 (0.51%)	~2.1-fold increase	16,720 $\pm$ 134	85.3

^a Measurements were performed in water at a constant temperature of 20 °C. The fluorescence quantum yields were determined relatively to curcumin ($\Phi = 0.011$).**Table 4**Analytical and photophysical performance of the SenNIR probe series for the detection of SA- β -gal.

Probe	Linker Architecture	λ_{abs} (nm)	λ_{em} (nm) ^a	Stokes Shift (nm)	Fold Enhancement ^b	LOD (mU)	Linear Range (mU)	Responsive Time	Cellular Contrast
SenNIR-1	meta-ether	425	688	263	~17-fold	0.49	0 – 150	< 8 min	Good differentiation
SenNIR-2	ortho-amide	435	690	255	~119-fold	0.4	0 – 400	< 5 min	Superior differentiation
SenNIR-3	ortho-ether	450	730	280	~9-fold	0.62	0 – 400	< 7 min	Good differentiation

^a Emission maximum recorded upon excitation at the optimal NIR-compatible wavelength.^b Fluorescence enhancement calculated at the respective NIR emission maximum following complete enzymatic cleavage.

electronic coupling within the fluorophore core.

Furthermore, the kinetic profile of the probe was monitored in the presence of 150 mU of the enzyme. Time-dependent fluorescence spectra revealed a steady increase in signal intensity over time upon excitation at both 465 nm and 585 nm. The resulting fluorescence intensity at 730 nm exhibited linear correlations with respect to time, confirming the probe's suitability for quantitative monitoring of enzymatic activity (Fig. 11C,D).

To observe the response rate of SenNIR-3 in the presence of β -gal kinetic fluorescent experiments were conducted by monitoring the fluorescence signal at 730 nm at different concentrations of the enzyme. According to Fig. 12A in the presence of 2.0 U the fluorescent signal of the probe achieves its peak value in 400 s. The selectivity of this probe towards different analytes including metals, amino acids and ROS was further evaluated via fluorescence spectroscopy by monitoring the ratiometric fluorescent signal (I_{730}/I_{535}). As illustrated in Fig. 12B, the ratiometric fluorescent signal was barely altered by the addition of any analytes (100 μ M) but β -gal (400 mU), demonstrating the great selectivity of our probe.

3.15. Cytotoxicity evaluation of SenNIR-3

SenNIR-3 was tested for its cytotoxic effect by MTT assay. Interestingly, unlike 1 and 2, SenNIR-3 exhibited a mild cytotoxicity at concentrations higher than 5 μ M after exposure of human normal fibroblasts for 24 or 48 h (Fig. 13).

3.16. Bioimaging of endogenous SA- β -gal in live cells

Subsequently, we investigated whether SenNIR-3 possesses the ability to visualize SA- β -gal in senescent cells as compounds SenNIR-1 and SenNIR-2, in a model of premature senescence. For that purpose normal untreated and H₂O₂-treated BJ cells were incubated with 5 μ M of SenNIR-3 and 1 mM of D-galactose in culture medium for 1 h at 37 °C. As illustrated in Fig. 14, SenNIR-3 staining showed a significant increase in H₂O₂-treated BJ cells.

3.17. Ex vivo bioimaging evaluation of SenNIR-3

Finally, SenNIR-3 was assessed, as compounds SenNIR-1 and 2, in sections from three main organs, i.e., heart, liver, and kidney from 5- and 24-month-old C57BL/6 J male mice. Our findings, as shown in Fig. 15, confirmed its capacity to stain SA- β -gal in aged tissues, as

sections from 24- months mice displayed higher levels of fluorescence in CSLM.

3.18. Comparative analysis of the spectral profiles and senescence selectivity of SenNIR analogues

A systematic comparison of the three architectures reveals that the regiochemical positioning and the chemical nature of the linker significantly modulate the optical properties and activation kinetics of the SenNIR platform. In all designs, the SA- β -gal responsive trigger was conjugated via a *para*-substitution pattern relative to the fluorophore's dicyanomethylene core to minimize steric hindrance and maximize electronic communication. The lysosomal morpholine unit was then evaluated at two distinct positions *ortho* and *meta* using either ether or amide linkages. SenNIR-1 features the targeting moiety attached via an ether bond at the *meta* position. This configuration produced dual emission profiles: 575 nm ($\lambda_{\text{exc}} = 465$ nm) and 688 nm ($\lambda_{\text{exc}} = 585$ nm). Upon enzymatic titration with the enzyme, a 37 nm bathochromic shift and a 3-fold fluorescence enhancement were observed at 465 nm excitation. Conversely, excitation at 585 nm yielded a ~17-fold increase in intensity at 688 nm, accompanied by a characteristic shoulder at approximately 770 nm.

SenNIR-2 employs an amide linkage at the *ortho* position. This structural modification proved to be the most responsive, exhibiting a superior fluorescence enhancement profile. Activation by the enzyme induced a 23 nm red-shift with a 54-fold intensity increase under 475 nm excitation. Most notably, excitation at 585 nm resulted in a remarkable ~119-fold fluorescence enhancement at 690 nm, with a pronounced shoulder appearing at 775 nm. This suggests that the *ortho*-amide configuration optimizes the ICT state efficiency upon trigger cleavage.

SenNIR-3 was designed as a hybrid architecture, utilizing an ether bond at the *ortho* position. This analogue exhibited the most significant spectral displacement. Upon excitation at 465 nm, the probe displayed a 134 nm bathochromic shift (from 582 nm to 716 nm), alongside an 8-fold enhancement. Under 585 nm excitation, a ~9-fold increase in fluorescence was recorded at 728 nm.

Collectively, these results demonstrate that while SenNIR-2 offers the highest sensitivity (highest fold-increase), SenNIR-3 provides the most optimized NIR window. This shift deeper into the NIR region (730 nm) is highly beneficial for maximizing tissue penetration depth (up to several millimeters), as longer wavelengths undergo substantially less scattering and avoid tissue absorbance from endogenous

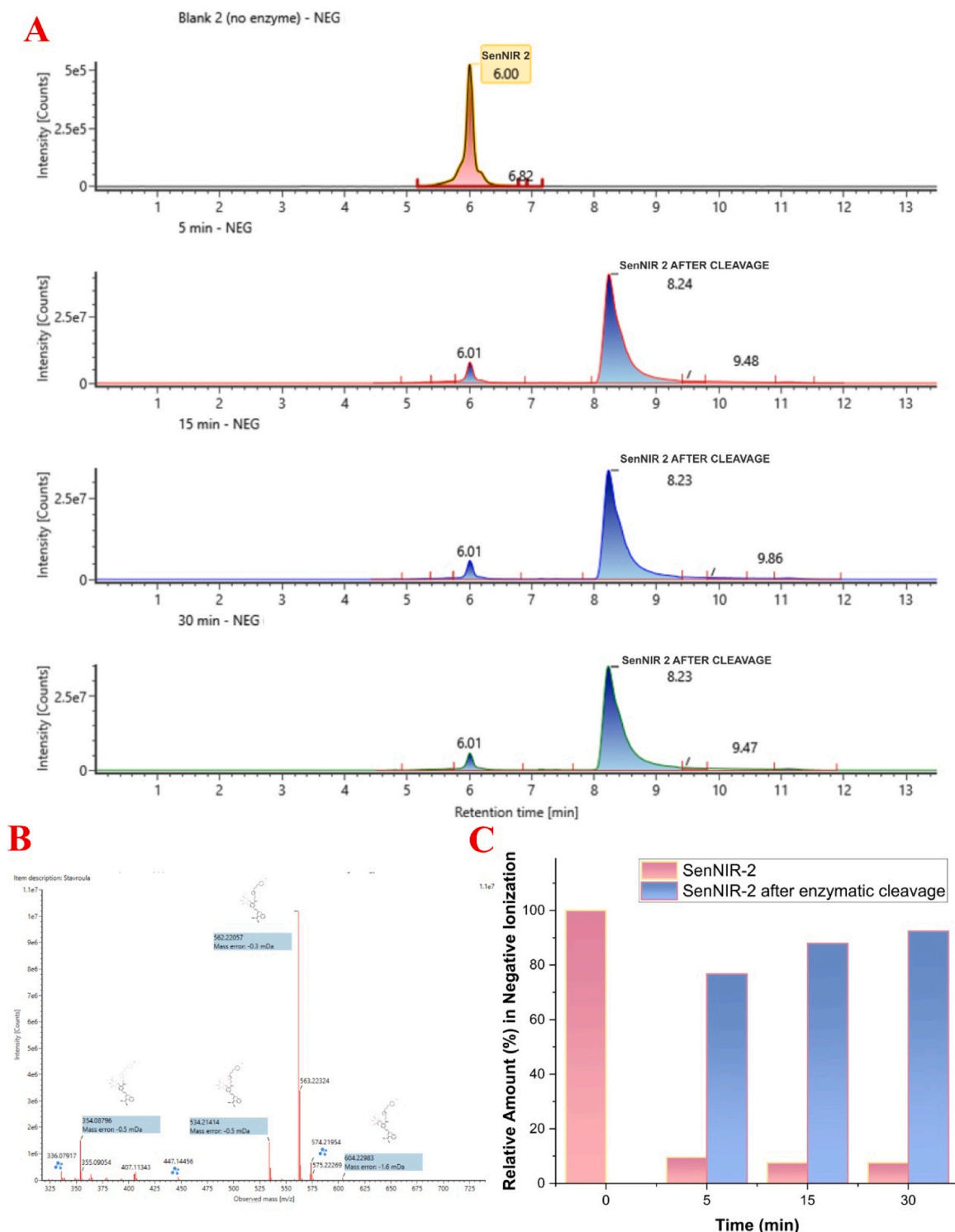
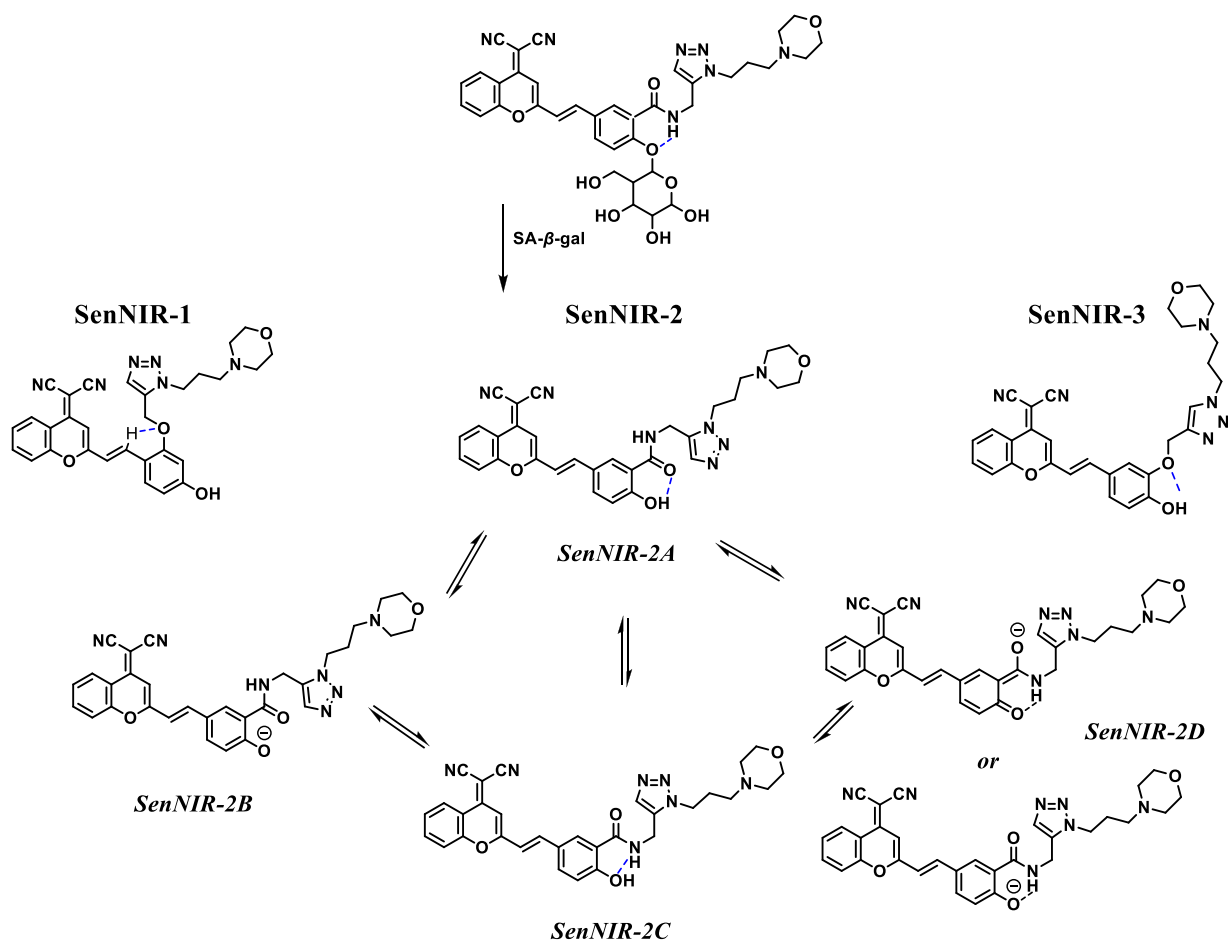


Fig. 16. Comprehensive LC-HRMS monitoring and relative quantification of the SenNIR-2 SA- β -gal cleavage. (A) Time-course chromatograms acquired in negative ionization mode at different incubation intervals (Blank/0 min, 5, 15, and 30 min). The traces clearly demonstrate the progressive consumption of the intact SenNIR-2 probe (retention time $\sim$ 6.01 min) and the simultaneous formation of the enzymatic cleavage product (retention time $\sim$ 8.23 min). (B) High-energy mass spectrum providing structural confirmation via characteristic fragmentation patterns, with highly accurate mass assignments and minimal mass errors. (C) Bar chart illustrates the relative amount (%) of the intact SenNIR-2 probe and its cleavage product over the 30-minute reaction period, highlighting the rapid kinetics and high efficiency of the enzymatic conversion.

chromophores. However, SenNIR-2 (emitting at 690 nm, which is already well within the effective deep-tissue NIR window) that stands out as the definitively superior diagnostic tool. Because effective tissue imaging relies not just on wavelength but also on overcoming background autofluorescence, SenNIR-2's exceptional $\sim$ 119-fold signal

enhancement provides a significant signal-to-noise ratio. This signal amplification ensures robust and highly sensitive detection in thick *ex vivo* specimens and potential *in vivo* models. This comparative study underscores how subtle variations in the topochemical arrangement of the targeting and triggering units can be exploited to fine-tune the



Scheme 6. Schematic representation of the calculated compounds.

performance of NIR agents for complex biological imaging (Table 2).

The distinct photophysical properties of the **SenNIR** compounds can be justified by the different molecular architectures utilized for combining the core moieties, which were designed to be common across all three structures. In all three molecules, the attachment of the galactosyl group via a glycosidic linkage converts the strongly electron-donating phenolic oxygen into a significantly weaker electron donor oxygen. This reduction in electron-donating capability disrupts the efficient ICT from the donor oxygen to the dicyanomethylene acceptor, thereby keeping the fluorescence quenched in the inactive state. However, in the presence of SA- β -gal, the sugar moiety is cleaved, realizing the highly electron-rich free hydroxyl group. This allows the electron density to flow efficiently through the conjugated π -system of the molecule towards the nitrile groups of the DCM core, fully restoring the ICT and turning on the NIR fluorescence. However, in the presence of SA- β -gal, the sugar moiety is cleaved, allowing the electron density to flow from the hydroxyl group through the conjugated π -system of the molecule towards the nitrile groups of the DCM core. Nonetheless, the linkage of the lysosome-targeting group can also influence the fluorescence of the molecule.

Starting with **SenNIR-1**, the targeting group is connected via an ether bond at the *meta* position relative to the hydroxyl group. The oxygen atom of the ether linkage can act as an electron donor; however, due to its *meta* position, it does not significantly impact the photophysical properties of the molecule.

In **SenNIR-3**, the same ether linkage is introduced, but this time at the *ortho* position relative to the -OH group. Since the *para* and *ortho* positions are electronically more influential, **SenNIR-3** exhibits enhanced photophysical properties, with an emission wavelength

extending up to 730 nm compared to 688 nm for **SenNIR-1**.

In **SenNIR-2**, the substitution also occurs at the *ortho* position, but the targeting group is linked through an amide bond. This bond introduces a carbonyl group, which, unlike an ether, acts as an electron acceptor group rather than an electron donor. Moreover, upon removal of the sugar moiety and restoration of the hydroxyl group, an intramolecular hydrogen bond can be formed, resulting in the formation of a low-strain, thermodynamically stable 6-membered ring. This hydrogen bonding effect is supported by NMR spectroscopy: in DMSO- d_6 at 25 °C and at the same pH, the hydroxyl proton of **SenNIR-1** appears as a singlet at 10.37 ppm (Figure S14), while in **SenNIR-3** it is observed as a broad peak in the range of 13.20–12.09 ppm (Figure S25).

A comparative evaluation of the photophysical properties across the **SenNIR** probes verifies their efficient enzymatic activation and structural robustness. Following the β -gal-mediated cleavage of the trigger moiety, a noticeable enhancement in both fluorescence quantum yield and emission intensity is observed. Specifically, **SenNIR-2** exhibits the highest relative quantum yield increase (~ 5.7 -fold), followed by **SenNIR-1** (~ 4.3 -fold) and **SenNIR-3** (~ 2.1 -fold) (Table S1). The inherent fluorescence quenching of the intact probes in aqueous media proves highly advantageous, as it effectively minimizes background signal interference prior to activation.

The molar extinction coefficients (ϵ) of the **SenNIR** probes were also evaluated. Before activation, the intact probes showed high ϵ values ($\sim 29,000$ – $30,000 \text{ M}^{-1} \text{ cm}^{-1}$), indicating strong light absorption in the near-infrared region. After enzymatic cleavage, these values dropped to about $16,700$ – $18,700 \text{ M}^{-1} \text{ cm}^{-1}$ (Figure S46). This decrease occurs because the removal of the trigger group changes the molecule's electronic structure. Importantly, this structural change is turning on

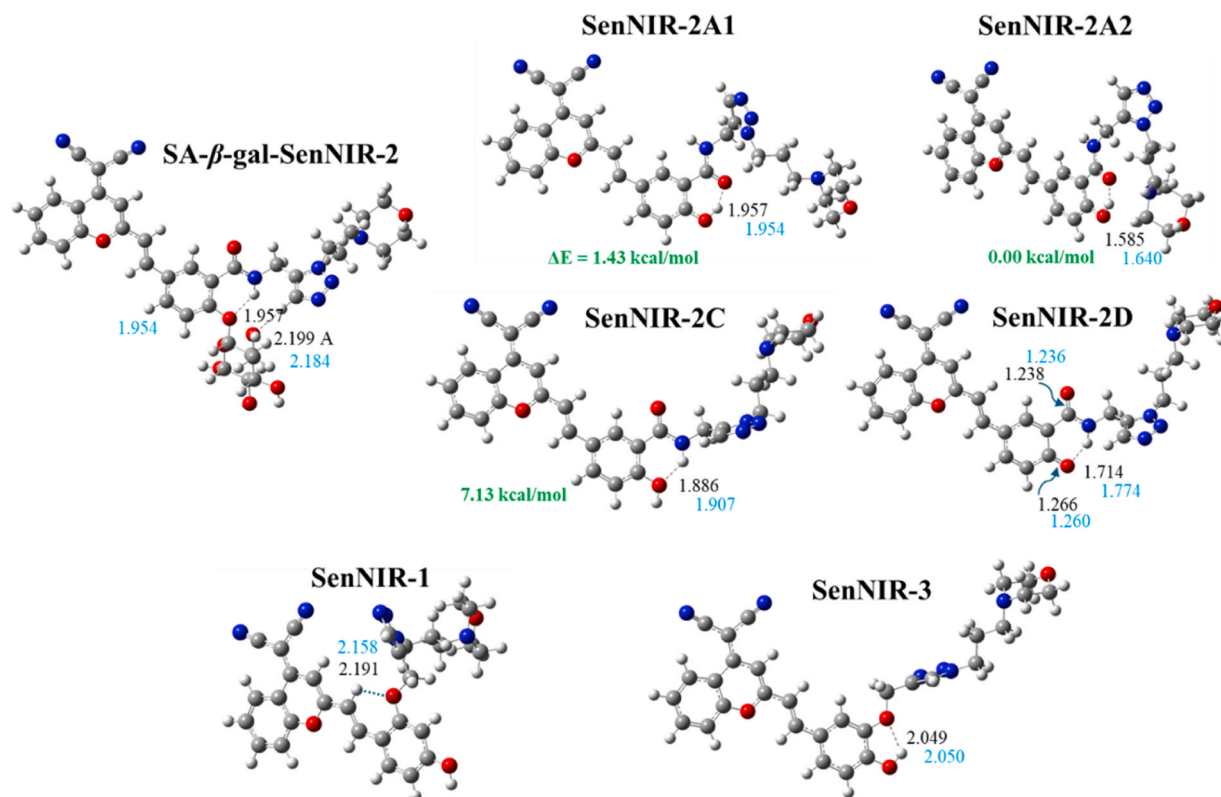


Fig. 17. Lowest in energy calculated compounds and isomers. Selected bond distances in Å of the S_0 states (black letters) and S_1 states are given (blue letters). Relative energies of the calculated **SenNIR-2** isomers are given in kcal/mol (green letters). [N: blue spheres, C: grey, H: white, and O: red].

Table 5

Summary of the main calculated and experimental λ (nm) absorption and emission UV-vis excitations.

		Calculated	Vibronic	EXPT	Calculated	Vibronic	EXPT
		before enzymatic cleavage			after enzymatic cleavage		
SenNIR-1	Absorption				651(CT), 439		450
	Emission				516		612/688
SenNIR-2	Absorption	743(CT), 419		435	521		512
	Emission	720(CT), 512	609(S_2)	685/690	603	670	708/690
SenNIR-3	Absorption				702(CT), 448		450
	Emission				526		716/728

fluorescence and leads to the increased quantum yield and overall optical brightness (Table 3).

Furthermore, fluorescence decay measurements reveal a consistent decrease in fluorescence lifetimes upon enzymatic cleavage, indicating a faster de-excitation pathway for the released fluorophores compared to their intact precursors (Figure S47 and Table S2). This distinct lifetime shift renders the probes highly compatible with fluorescence lifetime imaging microscopy, with **SenNIR-3** demonstrating optimal performance due to its superior lifetime contrast (>1 ns difference between the intact and cleaved states). Finally, continuous irradiation assays confirm the excellent photostability of the probes at low irradiation power (e.g., 5 mW). While slight photobleaching is detected at significantly higher power levels (up to 50 mW), the overall photophysical profile underscores the remarkable resilience and suitability of the **SenNIR** platform for demanding biological imaging applications (Figure S48).

A comprehensive summary of the photophysical and analytical parameters for the **SenNIR** series is provided in Table 4, illustrating how the structural variations directly dictate sensing performance.

To further validate the enzymatic activation mechanism, LC-HRMS kinetic assays were conducted across the entire series of probes

(**SenNIR-1**, **SenNIR-2**, and **SenNIR-3**). As explicitly detailed for **SenNIR-2** (Fig. 16), the cleavage was monitored over a 30-minute incubation period. The kinetic analysis revealed a remarkably rapid enzymatic response. Within just 5 min of incubation, the intact probe was almost entirely consumed, accompanied by the concurrent emergence of the cleavage product as the predominant species. Relative quantification demonstrated that the enzymatic conversion was highly efficient, reaching near-completion by the end of the 30-minute monitoring period, thereby confirming the rapid activation of the probes (Figure S49–51).

3.19. Computational insights into molecular geometry and photophysical performance

DFT calculations were employed to elucidate the structural features governing the performance of the **SenNIR** analogues. The computational data provide optimized geometries and predict that for all three compounds (**SA- β -gal-SenNIR-2**, **SenNIR-1**, **SenNIR-2** and **SenNIR-3**) intramolecular hydrogen bonds (IHBs) are established in their respective global minimum structures (Scheme 6 and Fig. 17). These IHBs play a pivotal role in maintaining the structural integrity and

Table 6Calculated absorption and emission excitations λ (nm), energy excitation ΔE (eV) and oscillator strength f ; our experimental values are also included.

		λ	ΔE	f	λ_{LR}	ΔE_{LR}	EXPT
Absorption^a							
SA-β-gal	$S_0 \rightarrow S_1$	447.9	2.768	0.000	743.0	1.669	
	$S_0 \rightarrow S_2$	418.6	2.962	1.432	409.3	3.029	435
SenNIR-2A1	$S_0 \rightarrow S_1$	449.0	2.761	0.000	721.7	1.718	
	$S_0 \rightarrow S_2$	430.1	2.882	1.257	422.8	2.933	512
SenNIR-2A2	$S_0 \rightarrow S_1$	476.9	2.600	0.005	693.2	1.789	
	$S_0 \rightarrow S_2$	427.8	2.898	1.257	420.2	2.950	
SenNIR-2C	$S_0 \rightarrow S_1$	448.4	2.765	0.000	736.4	1.684	
	$S_0 \rightarrow S_2$	418.5	2.963	1.298	409.0	3.031	
SenNIR-2D	$S_0 \rightarrow S_1$	521.4	2.378	1.375	504.6	2.457	
	$S_0 \rightarrow S_2$	402.1	3.084	0.000	609.7	2.034	
SenNIR-1	$S_0 \rightarrow S_1$	453.1	2.736	0.000	650.5	1.906	
	$S_0 \rightarrow S_2$	439.4	2.822	1.145	432.8	2.865	450
SenNIR-3	$S_0 \rightarrow S_1$	437.3	2.835	0.001	701.7	1.767	
	$S_0 \rightarrow S_2$	448.0	2.767	1.193	450.9	2.750	450
Emission^b							
SA-β-gal	$S_1 \rightarrow S_0$	720.0	1.722	0.000			685/690
	$S_2 \rightarrow S_0$	511.6	2.423	1.711			
SenNIR-2A1	$S_1 \rightarrow S_0$	516.4	2.401	1.634	489.2	2.534	708/690
SenNIR-2A2	$S_1 \rightarrow S_0$	945.9	1.311	0.001			
	$S_2 \rightarrow S_0$	497.7	2.491	1.555			
SenNIR-2C	$S_1 \rightarrow S_0$	505.9	2.451	1.653	489.2	2.534	
SenNIR-2D	$S_1 \rightarrow S_0$	603.3	2.055	1.533	549.5	2.256	
	vibronic				670/710		
SenNIR-1	$S_1 \rightarrow S_0$	515.9	2.404	1.621	487.4	2.544	612/688
SenNIR-3	$S_1 \rightarrow S_0$	526.4	2.55	1.628	475.5	2.608	712/728

^a Absorption vertical excitations: λ_1 ($S_0 \rightarrow S_1$; H $\rightarrow$ L) and λ_2 ($S_0 \rightarrow S_2$; H-1 $\rightarrow$ L). ^b Emission vertical excitations: λ_1 ($S_1 \rightarrow S_0$) and λ_2 ($S_2 \rightarrow S_0$).

planarity of the fluorophore scaffold, which is essential for efficient electronic communication.

The exceptional 119-fold fluorescence enhancement observed for **SenNIR-2** upon enzymatic activation is mechanistically explained by a strategic reconfiguration of its electronic and structural landscape. In general, fluorescence enhancement can occur through two opposite mechanisms: (1) the intramolecular charge transfer (ICT) mechanism and (2) loss or decrease of the CT character. The first case is an ICT-based typical turn-on probes, where the deprotection creates/restores a donor, the CT character is enhanced and leads to fluorescence. For instance, protonation can make the molecule rigid, which enhances ICT character and ultimately leads to fluorescence enhancement [78,79]. In the second case the post-cleavage loses CT character, the fluorescence is restored. The CT states have low oscillator strength and fluorescence is quenched. When the CT character is lost, the de-excitation has a higher oscillator strength and thus the fluorescence is enhanced [80–82].

Computational data reveal that the *ortho*-amide linkage leads to the formation of a highly rigid, planar global minimum (**SenNIR-2A2**) stabilized by an intricate network of six-membered intramolecular hydrogen bonds. This structural rigidification significantly suppresses non-radiative deactivation pathways. DFT calculations demonstrate a unique reversal of the frontier molecular orbital ordering in the activated deprotonated species (**SenNIR-2D**). Unlike the structural analogues, the $S_1 \rightarrow S_0$ de-excitation in **SenNIR-2D** loses the charge-transfer (CT) character, thus, the fluorescence enhancement occurs via the second mechanism mentioned above. Note that the CT de-excitation is a low-intensity process, and the radiative rate is suppressed. This transition from a CT, where the radiative rate is suppressed, to a highly radiative non-CT state, which is locally excited, combined with strong vibronic coupling that shifts the emission into the high-transparency NIR window (~ 710 nm), provides a comprehensive rationale for the superior sensitivity and 'turn-on' efficiency of the **SenNIR-2** platform (Table 5).

More specifically, conformation analysis for the compounds of Scheme 6 has been carried out. **SenNIR-2B** is not stable and it is easily converted to **SenNIR-2D**. Both S_0 and S_1 states of all calculated minimum structures are similar, see Figures S52–82 of SI. The S_0 optimized structures are given in Fig. 17, where selected bond distances of both S_0

and S_1 states are given. In all calculated structures, six-membered rings via van der Waals hydrogen bonds are formed, except for **SenNIR-3**, where a five-membered ring is formed which further stabilizes the compounds. The strong hydrogen bonds are formed that range from 1.585 Å to 2.199 Å. The relative energies of the calculated **SenNIR-2A1**, **-2A2** and **-2C** are given in Fig. 17. In the **SenNIR-2A2** structure, global minimum, the group on the right of the carbonyl bond is folded, to interact with the formed six-membered ring due to hydrogen van der Waals bond -OH...O and thus it is slightly more stable than unfolded **SenNIR-2A1** by 1.43 kcal/mol. In the **SenNIR-2C**, the six-membered ring is formed via the O...HN bond; the structure is 7.13 kcal/mol higher in energy, showing that the inter ring is favored via the O atom of the carbonyl group. In the deprotonated **SenNIR-2D** structure, where a six-membered ring is formed via the O...HN bond, both C-O bonds have similar bond distances indicating that both bonds are double, the C=O of the carbonyl group is only 0.03 Å shorter than the C=O bond of the phenyl group, see Fig. 17. Furthermore, the charge of the corresponding O atoms is -0.61 (carbonyl group) and -0.67 e⁻ for both S_0 and S_1 states. Thus, both **SenNIR-2D** exist simultaneously.

IR spectra of the compounds have been calculated. In the case of the **SenNIR-2D** the highest vibrational peak exists at about 1530 cm^{-1} and it corresponds to a normal mode that is characterized by bond stretching of the molecule including the C-N of the carbonyl group, see Figure S59–S60 of SI. Finally, the ^1H NMR spectrum of the calculated compounds was calculated, see Figure S61–S62 of SI, where a good agreement with the experimental values is observed. The hydrogen bonding effect is observed in the ^1H NMR spectra, the hydroxyl proton of **SenNIR-2A** appears at 14.5 ppm, while amine proton in **SenNIR-2D** appears at 12.4, see Figure S62 of SI.

The UV–vis absorption and emission spectra of the studied structures were calculated, while the cLR corrections were applied to include non-equilibrium solvent effect, see Table 6. cLR is important in the cases of CT excitations, and it improves the accuracy for vertical excitations in polar solvents. The absorption vertical excitations $S_0 \rightarrow S_1$ (λ_1) and $S_0 \rightarrow S_2$ (λ_2) correspond to H $\rightarrow$ L and H-1 $\rightarrow$ L molecular orbital transition. In all calculated structures, apart from **SenNIR-2D**, the H $\rightarrow$ L excitation corresponds to an charge transfer excitation from oxazinane group to dicyanomethyl group and naphthalene-O analog group, while the

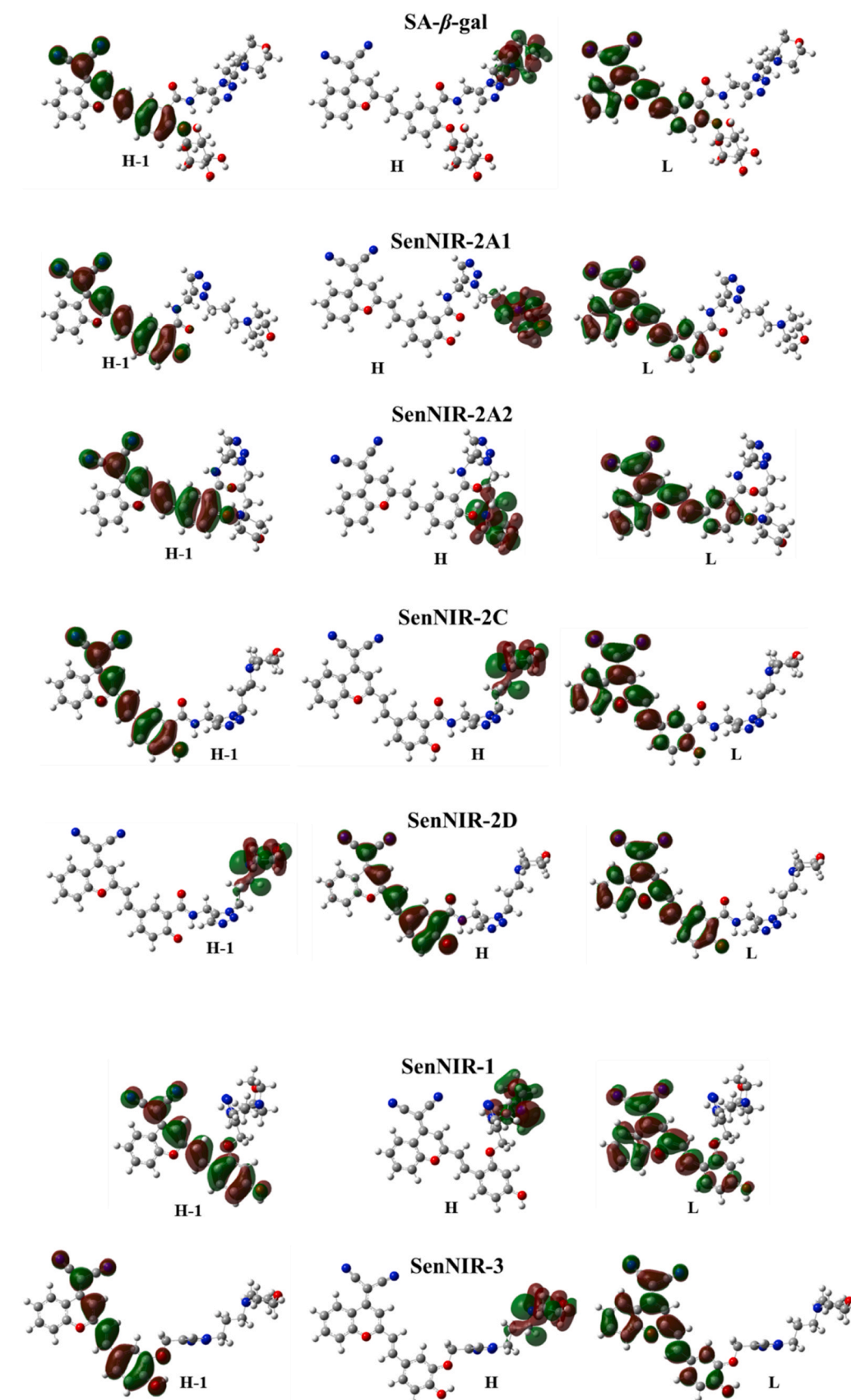


Fig. 18. Frontiers molecular orbitals (HOMO-1, HOMO, and LUMO) of the SA-β-gal-SenNIR-2, SenNIR-2, SenNIR-1 and SenNIR-3 at PBE0/6-31 G(d, p) method.

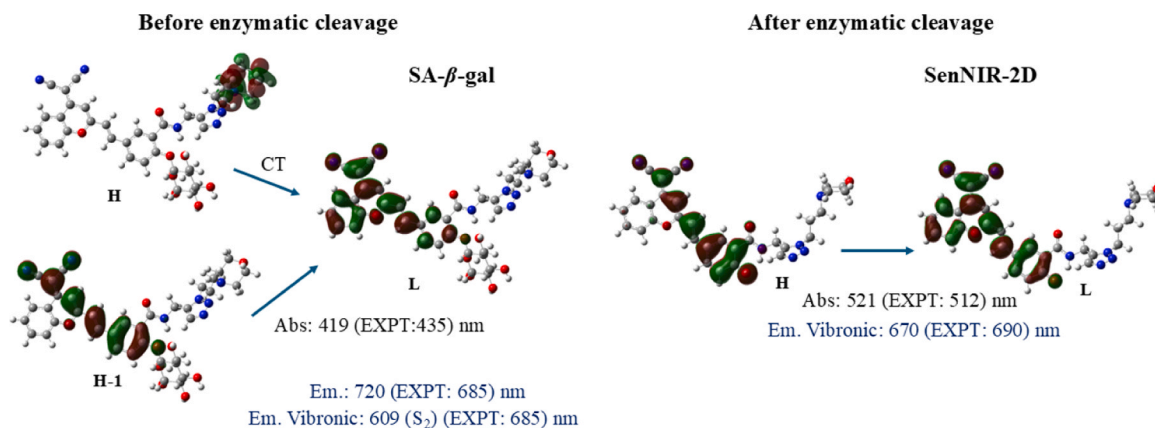


Fig. 19. Leading frontiers molecular orbitals of the SA-β-gal-SenNIR-2 and SenNIR-2 involved in the main calculated absorption and emission peaks at the PBE0/6-31 G(d,p) method.

H-1→L excitation is not a charge transfer excitation, only a small delocalization is observed regarding the phenyl group of the naphthalene-O analog group, see Fig. 18. On the contrary, the SenNIR-2D has reversed H-1 and H molecular orbitals, with respect to the other calculated structures. Overall, there is good agreement between the experimental and computational absorption λ values; the difference ranges between 1 nm to 16 nm. Regarding SenNIR-2, the lowest in energy structure SenNIR-2A2 presents the vis main peak at 428 ($\lambda_{LR}=420$) nm, while the protonated structure SenNIR-2D has the vis main peak at 521 ($\lambda_{LR}=505$) nm. The corresponding experimental value is at 512 nm, showing that in solvent mainly the deprotonated SenNIR-2D exist.

Regarding the emission spectra, it is observed that depending on the applied excited λ_{exc} , differences are observed in the experimental λ_{em} values up to 76 nm; this is indicative of the importance of the vibrational levels of the excited and the ground states. Thus, it seems that apart from electronic calculated UV-vis emission spectra, the vibronic excitations should also be studied. For the SenNIR-2D spectra, the calculated λ_{em} value found at 603 ($\lambda_{LR}=550$) nm, while the 0-0 transition between the two lowest vibrational levels is calculated at 609 nm. At the vibronic emission spectra peaks at 670 nm and 710 nm are observed in good agreement with our experimental emission spectra at 708 nm or 690 nm depending on the applied excited λ_{exc} .

Comparing the photophysical properties of SA-β-gal-SenNIR-2 with the SenNIR-2D we conclude that for SA-β-gal-SenNIR-2, the $S_1 \rightarrow S_0$ correspond to a charge transfer deexcitation and the deexcitation route $S_2 \rightarrow S_1 \rightarrow S_0$ occurs. Note that the pre-cleavage CT state is dark, i.e. it's non-emissive with very low oscillator strength because the donor and acceptor orbitals are spatially separated, with a poor overlap and low transition dipole moment. For the SenNIR-2, the $S_1 \rightarrow S_0$ is not a charge transfer deexcitation explaining the experimental observation of the ~119-fold increase in emission peak intensity upon addition of β-gal. The vibronic coupling, combination of electronic and vibrational transitions, is important and shifts the spectra to lower-energy regions around ~700 nm.

The key property to predict compounds with appropriate photophysical properties so as to be used as NIR fluorescent and colorimetric probe, is to find a pair of compounds, i.e., initial compound (reactant) and final compound (product upon the application of the enzyme), where the initial compound presents H→L molecular excitation that correspond to a charge transfer excitation in NIR area while the H-1→L molecular excitation is in vis area and without having a charge transfer character. Upon the addition of the specific enzyme, the ordering of the excitation should be reversed, i.e., the H-1→L molecular excitation should present a charge transfer character, while the H→L should not.

Prior to enzymatic cleavage, the absorption profile is characterized by an $S_0 \rightarrow S_1$ excitation with distinct CT character (Fig. 18), while the

$S_0 \rightarrow S_2$ transition corresponds to the primary observed peak. Following enzymatic cleavage, a fundamental change is observed: both the $S_0 \rightarrow S_1$ absorption and the $S_1 \rightarrow S_0$ emission lose their CT character (Figs. 18 and 19); the locally excited states present high radiative rate. High oscillator strength is observed enabling efficient photon emission. This transition away from a charge-transfer de-excitation state provides a clear mechanistic explanation for the experimentally observed ~119-fold increase in emission intensity upon the addition of β-gal.

4. Conclusions

Herein, we developed SenNIR, a family of ratiometric, lysosome-targeted NIR fluorescent probes capable of imaging senescence through monitoring SA-β-gal activity in cells and tissues. Upon sensing senescence, these probes undergo a rapid and highly sensitive fluorescence enhancement, enabling precise visualization of senescent cells. The probes exhibited strong NIR fluorescence selectively in stress-induced senescent human diploid fibroblasts, while remaining inactive in proliferating cells. Moreover, they demonstrated no cytotoxicity and showed age-dependent signal enhancement in *ex vivo* tissue sections, including heart, liver, and kidney, highlighting their potential for *in vivo* aging studies. To assess the robustness of the sensor design, structural analogues (SenNIR-1, SenNIR-2, and SenNIR-3) with variations in linker type and substitution patterns were synthesized and evaluated. These studies revealed that regiochemical positioning and the chemical nature of the tethering moiety profoundly determine the photophysical properties, including emission maxima and fluorescence quantum yields. Specifically, transitioning from a *meta*-ether linkage (SenNIR-1, ~17-fold enhancement) to an *ortho*-amide linkage (SenNIR-2) dramatically boosted the sensitivity, yielding an exceptional ~119-fold fluorescence turn-on. Conversely, employing an *ortho*-ether linkage (SenNIR-3) resulted in a modest ~9-fold enhancement but provided the deepest NIR emission. We conclude that ideal NIR fluorescent probes require an initial compound with charge-transfer H→L (NIR) and non-charge-transfer H-1→L (vis) excitations, which reverse upon enzymatic reaction to enable strong turn-on emission. Collectively, our results underscore the diagnostic value and design flexibility of this platform. SenNIR and its analogues offer a powerful toolset for senescence imaging in both basic and translational research, with potential implications in aging, cancer, and age-related pathologies.

CRediT authorship contribution statement

Eleni Patsea: Resources. Aimilia D. Sklirou: Methodology, Investigation. Dimitrios A. Diamantis: Writing – original draft, Methodology, Investigation. Despoina D. Gianniou: Writing – review & editing, Methodology, Investigation. Stavroula G. Kyrkou: Writing – review &

editing, Writing – original draft, Validation, Methodology, Investigation. **Andreas G. Tzakos**: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Ioannis P. Trougakos**: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition. **Demeter Tzeli**: Writing – review & editing, Validation, Software, Methodology, Investigation, Data curation, Resources, Visualization. **Eleftherios Paraskevas Tzakos**: Writing – review & editing, Investigation. **Vassilis Zoumpourlis**: Resources.

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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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.snb.2026.140558](https://doi.org/10.1016/j.snb.2026.140558).

Data availability

Data will be made available on request.

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Stavroula G. Kyrkou is a PhD candidate in Chemical Biology at the University of Ioannina, where she also earned her bachelor's degrees in chemistry and Master's degrees in "Synthetic Chemistry and Biochemistry". Her doctoral research focuses on the development of novel theranostic agents and potent NIR probes for photodynamic therapy. Her expertise covers the design and synthesis of small organic molecules, the evaluation of their photophysical properties, and their characterization using NMR spectroscopy and Mass Spectrometry (MS). Currently, her work centers on engineering responsive probes for advanced biosensing and targeted therapeutic applications.

Dr. Despoina Gianniou is a molecular biologist and postdoctoral researcher at the Department of Biology, National and Kapodistrian University of Athens, Greece. She obtained her PhD in 2023, focusing on proteostatic mechanisms involved in tumorigenesis and aging. Her research centers on cancer biology, biomarker discovery and the molecular mechanisms underlying human diseases. She has extensive experience in multidisciplinary research projects, including EU-funded programs on natural bioactive compounds and clinical studies. Dr. Gianniou has co-authored 20 peer-reviewed publications and has presented her work at numerous international conferences.

Dr. Dimitrios A. Diamantis is a graduate of the Department of Chemistry at the University of Ioannina (2010–2014), specializing in Organic Chemistry. During the period 2014–2017, he completed his Master's thesis entitled "Modification of natural products aiming to enhance their bioavailability" at the Department of Chemistry of the University of Ioannina. From 2017–2022, he conducted his doctoral dissertation entitled "Rational design and synthesis of near-infrared (NIR) emitting diagnostic and theranostic compounds targeting the cancer cell microenvironment." He has acquired extensive experience in modern purification and identification methods. During his postgraduate and doctoral studies, he published 10 articles in international peer-reviewed journals.

Dr. Aimilia D. Sklirou is a Biologist holding a PhD in Biological Sciences from the National and Kapodistrian University of Athens. She has more than 12 years of experience in the field of Biomedicine and has worked extensively on studying the impact of proteomic instability on carcinogenesis. Furthermore, her research has focused on evaluating the potential biological activity of a wide variety of natural or synthetic products (e.g., crude extracts, micro-fractions, or pure compounds) derived from various biosphere sources, both *in vitro* and in human cell models, through her participation in 10 national or European Union-funded research programs. Dr. Sklirou is experienced in various molecular and biochemical techniques and methods, such as *in vitro* cell culture maintenance, nucleic acid handling (DNA and RNA isolation), gene amplification via PCR and qPCR, gene cloning and mutagenesis, agarose and acrylamide gel electrophoresis, Western Blotting, immunohistochemistry, biochemical techniques using fluorometry and spectrophotometry, as well as confocal microscopy. She has published a significant number of articles (40) in high-impact journals and chapters in international books and is actively involved in presentations at national and international conferences.

Eleni Patsea is a Pathologist and the Director of the Pathology Department at Metropolitan General, with extensive clinical experience in the field of diagnostic pathology.

Zoumpourlis Vassilis is a Director of Research, Head of the research team of Biomedical Applications Unit, Institute of Chemical Biology, National Hellenic Research Foundation. Dr. Vassilis Zoumpourlis is Director of Research and Head of the Biomedical Applications Unit within the Institute of Biology, Medicinal Chemistry and Biotechnology of the NHRF. He has been a Researcher Grade A since 2013 and is also head of the certified NHRF animal facility. He obtained his BSc in Molecular Biology and Genetics at University of J E Purkyne, Brno, Czech Republic in 1987, his M.Sc. from the same University in 1988 and his Ph.D. from the Medical School of Crete in 1994. She has practiced research in top Institutions in Europe and USA. In 1992–3 he served as a visiting Researcher in the Molecular Oncology Group in The University of Liverpool, UK, in 1995 in Marie Curie Institute, Paris, France, in 1998 in Sylvius Laboratory, Leiden, Netherlands, in 2002 in the Department of Molecular Biology, Amsterdam, Netherlands. Since 2004 he has also worked in the Mazaryk Institute, Brno, Czech R, in Paterson Institute, Cancer Research, Manchester, UK, & Roy Castle Lung Cancer Institute, The University of Liverpool, UK, as well as in the Comprehensive Cancer Center, University of California, San Francisco, USA. He has supervised 13 diplomas, 28 Master thesis, 26 PhD thesis and 14 Post-Doc Researchers. Since

2004 he is a Lecturer at Msc classes, Universities of Athens, Thessaloniki, Thessaly, Patras, Thrace and Crete. The topic of his lectures is based on the molecular mechanisms of cancer and stem cell biology. The interests of Dr Vassilis Zoumpourlis cover the field of the molecular biology of cancer, mainly the study of genes with a crucial role in the molecular mechanisms of multistage carcinogenesis (initiation, promotion, progression) and metastasis. Recently he has been actively involved in intriguing research of stem cells and their applications in cancer therapeutics and drug toxicity screening. The current challenge is to develop new generation translational products for cytotherapy-based cancer management and testing of drugs. To this end, Dr Vassilis Zoumpourlis group has recently transferred its long-term experience and know-how in molecular biology of cancer in the emerging field of stem-cell biology, in order (a) to design stem cell-based vehicles for effective and specific tumour targeting and (b) to develop improved *in vitro* preclinical assays for quick and reliable assessing of toxicology and safety profiles of drugs. Dr. Zoumpourlis maintains an impressive research record with 136 publications in international peer-reviewed journals, boasting a total Impact Factor exceeding 900. His work has garnered 9700 citations with an h-index of 47 (as of February 2026), while he has also contributed 182 papers to international and Greek conference proceedings. Throughout his 35-year career, he has secured more than 5 M€ in research funding from numerous national and international grants. His academic excellence has been recognized through various prestigious honors, including several scholarships from the University of J.E. Purkyne, the Fulbright Award (2007–2008) for research at UCSF, the 1st Galinos Prize, and multiple awards from the University of Athens, the Hellenic Pneumonological Society, and the International Kallikrein Symposium in Germany.

Eleftherios Paraskevas Tzakos holds a degree in Computer Science and a master's degree in bioinformatics from the Department of Biology of the National and Kapodistrian University of Athens (NKUA). He also holds a master's degree in digital marketing from Democritus University of Thrace (formerly the International Hellenic University). He works as a Senior Software Engineer and Scrum master's in research and development (R&D) at Nokia, with more than 17 years of experience in telecommunications systems and software development. He has contributed to scientific publications and collaborative research initiatives that integrate computational and biomedical approaches and has participated in the development of several software systems and in international patent activities.

Demeter Tzeli is Professor of Physical Chemistry, Chemistry Department, National and Kapodistrian University of Athens and Collaborating Researcher at Theoretical and Physical Chemistry Institute, National Hellenic Research Foundation, Athens, Greece. She has been the recipient of the Greek Award 2009 L'OREAL-UNESCO for the Women in Science. She is the author of more than 130 research articles in international journals. She is a reviewer of European and national organizations and member of the Editorial Boards of 8 scientific journals. She has expertise in Computational Chemistry and Physics. Her research includes computational studies of molecular systems ranging from diatomics to nanomaterials and proteins.

Ioannis Trougakos obtained his Ph.D. in Cellular-Developmental Biology from the National and Kapodistrian University of Athens (NKUA), Greece. He has worked as Post-Doc at EMBL, Germany, CBM "Severo Ochoa", Spain and at NHRF, Athens, Greece. Prof. Trougakos was elected Research Lecturer at NHRF and currently serves as Professor at the Faculty of Biology, NKUA where he (among others) leads the "Ageing and Age-Related Diseases" research group (<http://scholar.uoa.gr/itrougakos>). He is also appointed Adjunct Professor of "Systems Biology of Ageing and Cancer" at the European University of Cyprus. Prof. Trougakos has published articles in high-ranking journals, chapters in international books; he is also co-inventor in several patents. His group is funded by private (GR, EU, USA) and public (GR, EU) entities; also, the group participates in contractual activities with the Industry.

Dr. Andreas G. Tzakos is Professor of Organic Chemistry and Head of the Section of Organic Chemistry & Biochemistry in the Department of Chemistry at the University of Ioannina (Greece). He leads the Chemical Biology Laboratory and directs the Bioanalytical and Analytical Centers, which operate under ISO/IEC 17025 accreditation. He carried out postdoctoral research as an EMBO Fellow at the MRC Laboratory of Molecular Biology (Cambridge, UK) and has received major competitive funding and distinctions, including Marie Curie, EMBO, FEBS, NATO, and the Leonidas Zervas Award. His contributions to the discovery of potent bioactive compounds have also been recognized by the Academy of Athens. His research focuses on the design and development of small molecules, peptides, and conjugates for diagnostics and therapeutics, tumor-targeted systems, and tumor-responsive drug-delivery platforms. In parallel, he develops new bioactive agents and enabling technologies aimed at antibiotic resistance and neurodegenerative diseases. Dr. Tzakos has authored more than 170 peer-reviewed international publications, written one academic book, and edited three books. He has contributed to the development of three software tools and serves on the editorial boards of several international journals. He also serves on the scientific advisory board of a pharmaceutical companies and is an inventor on eight patents covering glioma-targeting delivery peptides, SPECT/PET theranostic platforms for glioma, and derivatization strategies for FDA-approved anticancer drugs to improve their activity profiles.