Supporting information

Bismuth-rhodamine: a new red light-excitable photosensitizer

Tasuku Hirayama,[a] Akari Mukaimine,[a] Kenta Nishigaki,[a] Hitomi Tsuboi,[a] Shusaku Hirosawa,[a] Kensuke Okuda,[a,b] Masahiro Ebihara,[c] and Hideko Nagasawa[a]

[a]Laboratory of Pharmaceutical and Medicinal Chemistry, Gifu Pharmaceutical University, 1-25-4, Daigaku-nishi, Gifu, 501-1196, Japan.

[b] Current adress: Laboratory of Bioorganic & Natural Products Chemistry, Kobe Pharmaceutical University, 4-19-1, Motoyamakita-machi, Hidashinada-ku, Kobe, Hyogo, 658-8558, Japan.

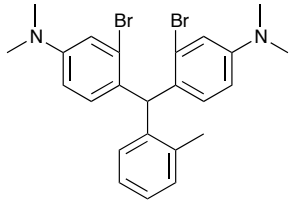
[c] Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, Yanagido, Gifu, 501-1193, Japan

Contents

General: All chemicals used in this study were commercial products of the highest available purity and were further purified by the standard methods, if necessary. ^{1}H-NMR spectra were obtained on a JEOLECA-500 spectrometer at 500 MHz and JEOL JNM AL-400 spectrometer at 400 MHz.^{13}C-NMR spectra were obtained on a JEOL ECA-500 spectrometer at 125 MHz and JEOL AL-400 spectrometer at 100 MHz. Chemical shifts of ^{1}H-NMR are referenced to tetramethylsilane (TMS). Chemical shifts of ^{13}C-NMR are referenced to $CDCl_3$ (77.0) or CD_3OD (49.0). Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. ESI-mass spectra were measured on a JEOL JMS-T100TD mass spectrometer. High-resolution mass spectra (HRMS) were measured on a JEOL JMS-T100TD by using polyethyleneglycol (PEG) as an internal standard. Reactions were monitored by silica gel TLC (Merck Silica gel 60 F_{254}) with visualization of components by UV light (254 nm) or with visual observation of the dye spots. Products were purified on a silica gel column chromatography (Taiko-shoji AP-300S).

1. Synthesis of BiR

4,4'-(*o*-tolylmethylene)bis(3-bromo-*N*,*N*-dimethylaniline) (1) [1]



To a solution of compound 3-bromodimethylaniline (2.00 g, 10.0 mmol) in 2 M aq.HCl (20 mL) was added 2-methylbenzaldehyde (601 mg, 5.0 mmol). After refluxing for 24 h, the reaction mixture was neutralized by $NaHCO_3$ and then extracted with CH_2Cl_2 (50 mL × 3). The combined organic layer was washed with brine (40 mL), dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography (silica gel, EtOAc / *n*-hexane) to afford the title compound as a white solid (1.14 g, 45%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.12–7.16 (m, 2H), 7.08 (t, *J* = 1.7, 7.5 Hz, 1H), 6.94 (d, *J* = 2.9 Hz, 2H), 6.73 (d, *J* = 7.5 Hz, 1H), 6.63 (d, *J* = 8.6 Hz, 2H), 6.54 (dd, *J* = 2.9, 8.6 Hz, 2H), 5.96 (s, 1H), 2.92 (s, 12H), 2.20 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 150.0, 141.7, 137.1, 131.1, 130.4, 129.6, 128.8, 126.6, 126.3, 125.7, 116.6, 111.3, 51.5, 40.5, 19.7.

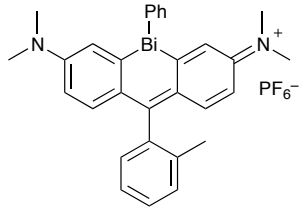
HRMS (ESI+) *m/z* calcd for $C_{24}H_{27}Br_2N_2$ $[M+H]^+$ 503.0516, found 503.0544.

Dichlorophenylbismuthine[2]

To a suspension of $BiCl_3$ (3.15 g, 10.0 mmol) in Et_2O (25 mL) was added a solution of triphenylbismuth (2.20 g, 5.00 mmol) in Et_2O (25 mL). The reaction mixture was stirred at room temperature for 6 h, and the solvent was evaporated. The crude solid was washed with Et_2O and dried in vacuo to give dichlorophenylbisumthine as a white powder (4.35 g, 81%). This material was used for the next step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.95–8.97 (d, *J* = 7.6 Hz, 2H), 7.88–7.92 (t, *J* = 7.6 Hz, 2H), 7.37–7.40 (t, *J* = 7.6 Hz, 1H).

BiR



A solution of 4,4'-(*o*-tolylmethylene)bis(3-bromo-*N*,*N*-dimethylaniline) **1** (900 mg, 1.80 mmol) in dry THF (10 mL) was cooled to −80 °C under an argon atmosphere. Then, *sec*-BuLi (4.5 mL, 4.5 mmol, 1.0 M in cyclohexane/*n*-hexane) was added dropwise. The mixture was stirred at −80 °C for 30 min. Then, the reaction mixture was slowly added via a syringe to a mixture of $PhBiCl_2$ (771 mg, 2.16 mmol) in dry THF (15 mL) at −80 °C. After stirring at the same temperature for 30 min, the mixture was warmed to room temperature. After stirring for 13 h, the reaction was quenched by 2 M HCl (100 μL), and the solvent was evaporated. The residue was diluted with MeCN (20 mL), and *p*-chloranil (885 mg, 3.60 mmol) was added. After stirring at room temperature for 2 h, KPF_6 (663 mg, 3.60 mmol) was added to the mixture. The resulting mixture was stirred at room temperature for 2 h. After evaporation, the residue was purified by column chromatography (silica gel, CH_2Cl_2 to CH_2Cl_2:MeOH = 10:1) to afford dark red solid (308 mg, ca. 75% purity, judged by ^{1}H-NMR spectrum). Recrystallization by slow diffusion of diethylether into a MeCN solution of the crude product provided pure **BiR** as a green crystal (51 mg, 4%). The crystals were used for X-ray single crystal analysis.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.75 (d, *J* = 6.9 Hz, 2H), 7.58 (d, *J* = 2.9 Hz, 2H), 7.43 (d, *J* = 9.7 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 1H), 7.26–7.33 (m, 4H), 7.20 (t, *J* = 7.5 Hz, 1H), 7.06 (d, *J* = 7.5Hz, 1H), 6.47 (dd, *J* = 9.8, 2.9 Hz, 2H), 3.29 (s, 12H), 1.80 (s, 3H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 178.8, 168.1, 157.1, 154.5, 143.5, 140.3, 136.7, 135.7, 130.9, 130.3, 129.3, 128.7, 128.0, 125.7, 125.1, 124.4, 113.5, 40.9, 19.2.

HRMS (ESI+) *m/z* calcd. for $C_{30}H_{30}BiN_2$ [M^+]: 627.2207; found 627.2201.

Anal. Calcd for $C_{32}H_{33}BiF_6N_3P$ [**BiR**+MeCN]: C, 47.24, H, 4.09; N, 5.16; found: C, 46.96; H, 4.09; N, 5.11.

Tetramethylrosamine[3]

N,*N*-dimethylaminophenol (400 mg, 2.92 mmol), 2-tolylaldehyde (178 mg, 1.46 mmol), and *p*-TsOH•H_2O (26 mg, 0.29 mmol) were dissolved in propanoic acid (10 mL). After stirring at 60 °C for 24 h, the mixture was poured into 3 M aq. NaOAc solution (100 mL). The mixture was extracted with $CHCl_3$ (50 mL × 3), and the combined organic layer was dried over $MgSO_4$ and then evaporated. The residue was dissolved with a mixture of $CHCl_3$ and MeOH (1:1, 40 mL). To the mixture, *p*-chloranil (356 mg, 1.46 mmol) was added. The resulting

mixture was stirred at room temperature for 18 h. After evaporation, the residue was purified by column chromatography twice (silica gel, 1st: $CHCl_3$:MeOH (6:1); 2nd $CHCl_3$:MeOH (5:1)) to afford tetramethylrosamine (17 mg, 2.3% as chloride salt). The obtained material was was dissolved in MeOH (5 mL), and a solution of KPF_6 (160 mg, 0.89 mmol) in MeOH (5 mL) was added. After stirring at room temperature for 1 h, the solvent was evaporated. The residue was taken up with $CHCl_3$ (10 mL), and the insoluble material was removed by filtration with a pad of celite. After evaporation, tetramethylrosamine (18 mg, 2.3%) was obtained as dark purple solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.51 (t, J = 8.2 Hz, 1H), 7.44–7.39 (m, 2H), 7.17–.15 (m, 3H), 6.90–6.87 (m, 4H), 3.33 (s, 12H), 2.04 (s, 3H).

2. X-ray data collection of BiR

A green block crystal of BiR obtained as above having approximate dimensions 0.33 × 0.31 × 0.28 mm^3 was mounted on a glass fiber. X-ray diffraction measurements were performed using a Rigaku AFC7R diffractometer equipped with a MoKα radiation (λ = 0.71075 Å) and a Rigaku Mercury CCD two-dimensional detector. Totally 12952 reflections were measured with the maximum $2\theta_{max}$ of 55.0°. All non-hydrogen atoms were refined anisotropically and all hydrogen atoms were treated as riding atoms. The crystal data and structure refinement results are summarized in Table S1. Crystallographic data for the structure of BiR have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 1530802.

3. Steady-state absorption and fluorescence spectroscopy

The UV-vis absorption spectra were recorded on an Agilent 8453 photodiode array spectrometer equipped with a UNISOKU thermo-static cell holder (USP-203). Fluorescence spectra were recorded using a JASCO FP6600 with a slit width of 5 nm and 6 nm for excitation and emission, respectively. To reduce fluctuation in the excitation intensity during measurement, the lamp was kept on for 30 min prior to the experiment. The path length was 1 cm with a cell volume of 3.0 mL. Quantum yields (Φ_{FL})were measured in 50 mM HEPES buffer (pH 7.4) by using a Quantaurus-QY absolute photo-luminescence quantum yields measurement system (C11347-01, Hamamatsu Photonics).

4. Cyclic voltammetry

Cyclic voltammetry was performed on a 600A electrochemical analyzer (ALS). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire as the auxiliary electrode, a glassy carbon electrode as the working electrode, and an Ag/Ag^+ electrode as the reference electrode. The sample solution contained 1 mM BiR or tetramethylrosamine and 0.1 M tetrabutylammonium perchlorate (TBAP) as a supporting electrolyte in MeCN, and argon was bubbled for 10 min prior to each measurement. The obtained potentials

were converted to those vs (Fc/Fc^+) by using ferrocene as an internal standard. The scan rate was 0.2 V s^{-1}.

5. Density functional theory (DFT) calculation

All calculations were performed by the density functional theory (DFT) calculations, using B3LYP exchange functional with LANL2DZ basis sets. The molecular orbital energy calculations were done by using Gaussian09 program.

6. Determination of quantum yield of BiR for $^1O_2$ generation and photostability tests

$^1O_2$ generation study was performed with an LED light source (625 nm, M625L3, Thorlabs) equipped with a liquid light guide (1.2 m, ϕ = 5 mm, LLG-0538-4, Thorlabs) and a T-cube light source driver (Thorlabs). Absorption spectra of mixed solutions of photosensitizer (BiR or methylene blue as a standard) and DPBF (diphenylbenzofuran, a colorimetric scavenger of $^1O_2$)[4,5] were measured as above every 1 min under irradiation (1.2 mW cm^{-2}). The quantum yield ($\Phi(^1O_2,)_{BiR}$) of BiR for $^1O_2$ generation was calculated from an initial photooxidation rate (k) of DPBF by using the equation as follows:

$$\Phi(^1O_2,)_{BiR} = \Phi(^1O_2,)_{MB} \bullet (k_{BiR}/k_{MB}) \bullet (F_{MB}/F_{BiR})$$

where MB means methylene blue as a standard photosensitizer, $\Phi(^1O_2,)_{MB}$ and F indicate quantum yield of methylene blue for $^1O_2$ generation (0.52 in MeCN)[4] and the absorption cofactor given by $F = 1-10^{-OD}$, respectively; OD is an optical density at irradiation wavelength. The initial absorption of DPBF at 410 nm was adjusted to 0.8, and those of BiR and methylene blue at 625 was adjusted to 0.1. For the photostability tests, absorption spectra BiR or methylene blue were acquired in the dark or under the irradiation condition without DPBF.

7. Cell culture experiments

Human hepatocellular carcinoma (HepG2) cells were cultured in modified essential medium (MEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% Antibiotic-Antimycotic (ABAM, Gibco), and 2 mM glutamine at 37 °C in a 5% CO_2/ 95% air incubator.

Human lung adenocarcinoma (A549) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% FBS, 1% ABAM, and 2 mM glutamine at 37°C in a 5% CO_2/ 95% air incubator.

Human embryonic kidney 293 cells (HEK293) cells were cultured in modified essential medium (MEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% Antibiotic-Antimycotic (ABAM, Gibco), and 2 mM glutamine at 37 °C in a 5% CO_2/ 95% air incubator.

Normal human diploid (TIG-3) cells were cultured in Eagle's minimal essential medium with L-glutamine and Phenol-Red (E-MEM, Wako pure chemicals) containing 10% fetal bovine serum (FBS, Gibco) and 1% Antibiotic-Antimycotic (ABAM, Gibco) at 37 °C in a 5% CO_2/ 95% air incubator.

Two days before use, cells (2.5×10^4) were seeded on Advanced TC glass-bottomed dishes (CELLview™ Cell

Culture Dish, Greiner).

8. Confocal fluorescence imaging experiments

Confocal fluorescence images were acquired with an Olympus IX83 microscope equipped with a 130 W mercury lump, an EMCCD camera (Hamamatsu Photonics, ImagEM), and a disk scan confocal unit (DSU). Images were obtained with appropriate filter sets for each dye as follows.

• BiR: excitation = 608–648 nm, emission = 672–712 nm, and dichroic mirror = 660 nm.

• ER-Tracker^{TM} green: excitation = 465–500 nm, emission = 516–556 nm, and dichroic mirror = 495 nm.

• Hoechst: excitation = 330–380 nm, emission = 460–480 nm, dichroic mirror = 400 nm.

• Propidium iodide (PI): excitation = 465–500 nm, emission = 572–642 nm, dichroic mirror = 562 nm.

For all imaging experiments, Hank's Balanced Salt Solution (HBSS, Gibco) containing calcium and magnesium without Phenol-Red was used. After washing the cells with HBSS (× 3), BiR (5 μM, from 1 mM stock solution in DMSO) was added. After incubation at 37 °C for 30 min, the cells were washed with HBSS, and then imaged.

For co-staining experiments, 500 nM ER-Tracker^{TM} green was used with 1 μM BiR in HBSS for 30 min. Image analysis was performed with ImageJ.

9. Cell death assay by PI/Hoechst staining

After incubation of cells with BiR for 30 min, the cells were washed with HBSS (× 2). The cells were irradiated with excitation light (608–648 nm, ca. 150 mW cm^{-2}) of the microscope for 1 min. Then, PI (final: 5 μM, from 500 μM stock solution in EtOH) and Hoechst 33342 (final: 1 ng/mL, from 0.1 μg/mL stock solution in water) were added to the cells. After incubation for 30 min, the cells were washed with HBSS and then imaged. The numbers of nucleus stained by PI and Hoechst were counted, and the cell death rate were evaluated by calculating the ratio of PI-positive cells/Hoechst-positive cells.

10. Cell death assay by MTT assay

The cells were seeded on 96-well cell plates (Techno Plastic Products AG) at 5.0 × 10^3 cells/well. The cells were incubated in the appropriate media for 24 h. Then, the cells were treated with various concentrations of BiR for 24 h under dark condition. Then, the cells were washed with the media and incubated with 0.5 mg mL^{-1} MTT reagent for 4 h. Then, the media was removed, and the cells were lysed with DMSO. Absorption at 570 nm of each wells were measured on a Multiskan JX plate reader (Thermo Fisher Scientific).

References

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<Figures>

Table S1. Crystallographic data and structure refinements for **BiR**

	BiR
Empirical formula	$C_{32}H_{33}BiF_6N_3P$
Formula weight	813.56
Crystal system	triclinic
Space group	$P\bar{1}$
a (Å)	10.7064(18)
b (Å)	11.8965(17)
c (Å)	14.505(2)
α (°)	66.593(6)
β (°)	77.962(6)
γ (°)	70.259(6)
V (Å^3)	1589.9(4)
Z	2
Temperature (K)	293
D_c (gm^{-3})	1.699
Absorption coefficient (mm^{-1})	5.657
F(000)	796
Crystal size (mm^3)	0.33 × 0.31 × 0.28 mm^3
Measured reflections	12952
Independent reflections	7179 [R_{int} = 0.0300]
Goodness-of fit on F^2	1.062
R [$I > 2\sigma$(I)]	R_1 = 0.0391, wR_2 = 0.0997
R (all data)	R_1 = 0.0458, wR_2 = 0.1065

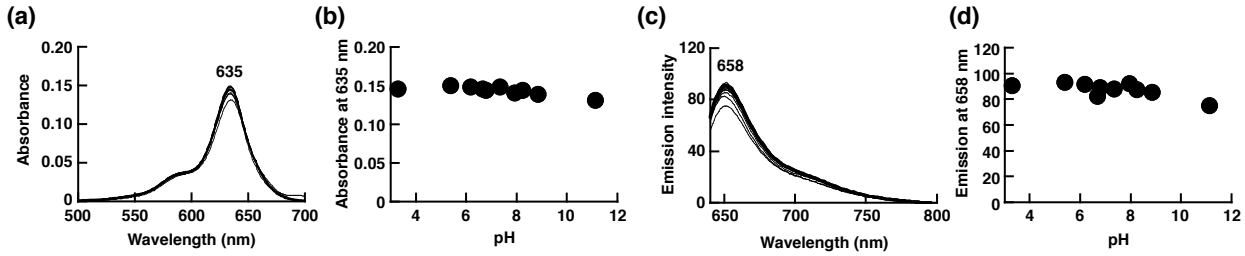


Fig. S1 pH titration of (a) absorption and (c) fluorescence of BiR (5 μM) in aqueous buffer at pH 3.3, 5.4, 6.2, 6.7, 6.8, 7.3, 7.9, 8.2, 8.9, 11.1. Each spectrum was measured after 30 min incubation. For fluorescence measurement, excitation was provided at 630 nm. (b) Plot of absorption at 635 nm of (a) against pH. (d) Plot of fluorescence intensity at 658 nm of (c) against pH.

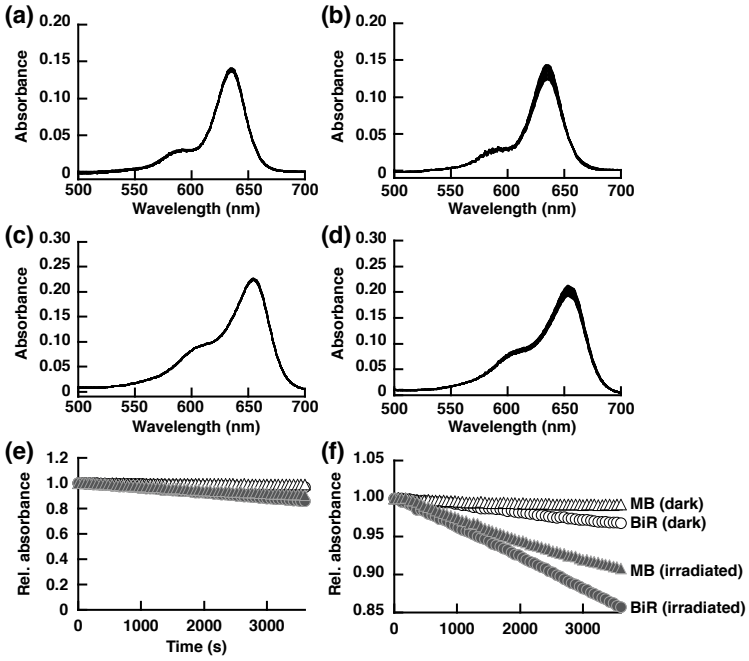


Fig. S2 Photostability tests of BiR and methylene blue (MB) as a reference. Absorption spectra of (a) BiR in the dark, (b) BiR under irradiation with 625 nm LED light (1.2 mW cm^{-2}), (c) MB in the dark, and (d) MB under the irradiation. Absorption spectra were acquired every 1 min for 1 h. (e) Time course plots of relative absorbance at the maximum (635 nm for BiR, 650 nm for MB) obtained in (a)–(d). Open circles: BiR, dark (a); Filled circles: BiR, irradiated (b); Open triangles: MB, dark (c); Filled triangles: MB, irradiated (d). (f) Magnified graph of (e).

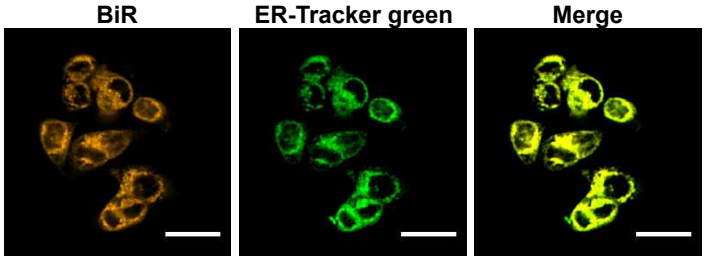


Fig. S3 Fluorescence images of HepG2 cells stained with 1 μM BiR and ER-Tracker Green. Left: Image obtained with a Cy5 filter (BiR). Middle: Image obtained with a FITC filter (ER-Tracker green). Right: Merged image of the left and middle images. Scale bars indicate 25 μm.

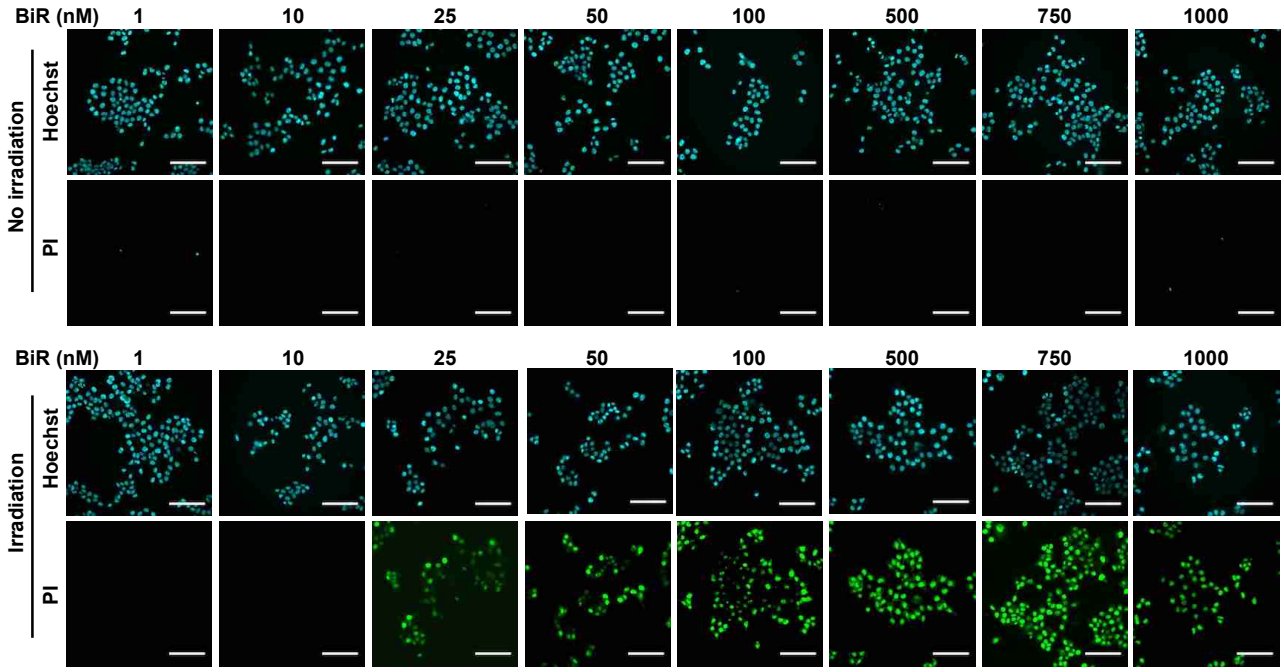


Fig. S4 Fluorescence images of HepG2 cells stained with PI and Hoechst without irradiation (upper) and with irradiation for 1 min in the presence of various concentration of BiR. Scale bars indicate 100 μm.

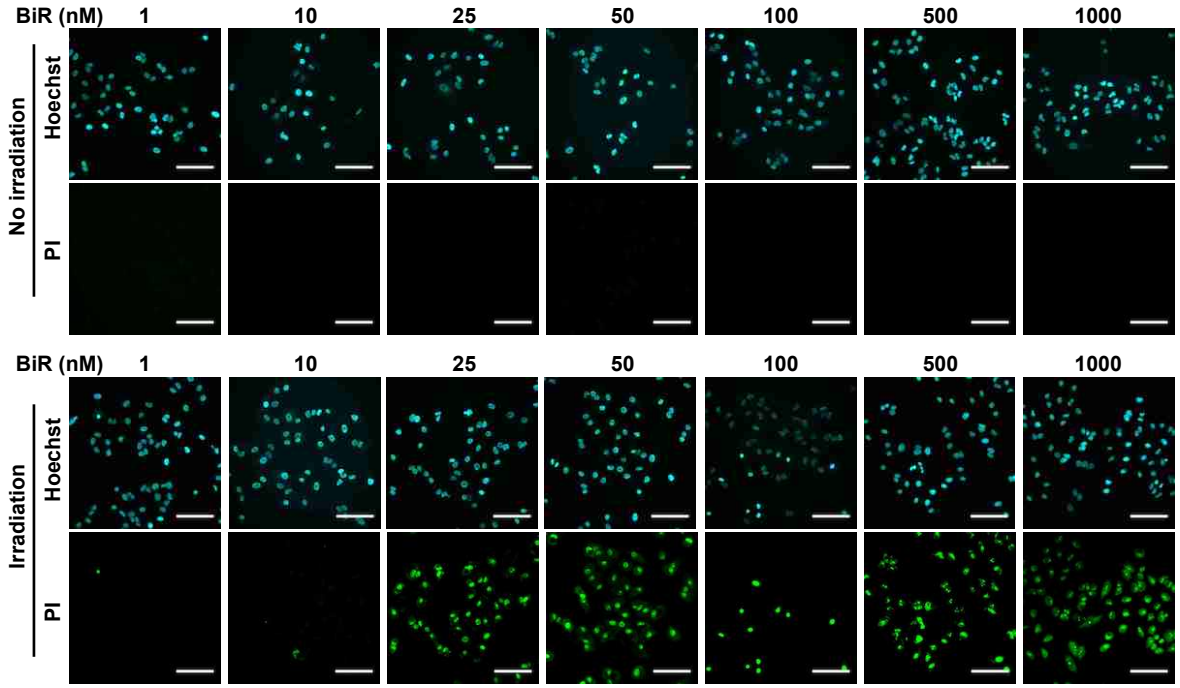


Fig. S5 Fluorescence images of A549 cells stained with PI and Hoechst without irradiation (upper) and with irradiation for 1 min in the presence of various concentration of BiR. Scale bars indicate 100 μm.

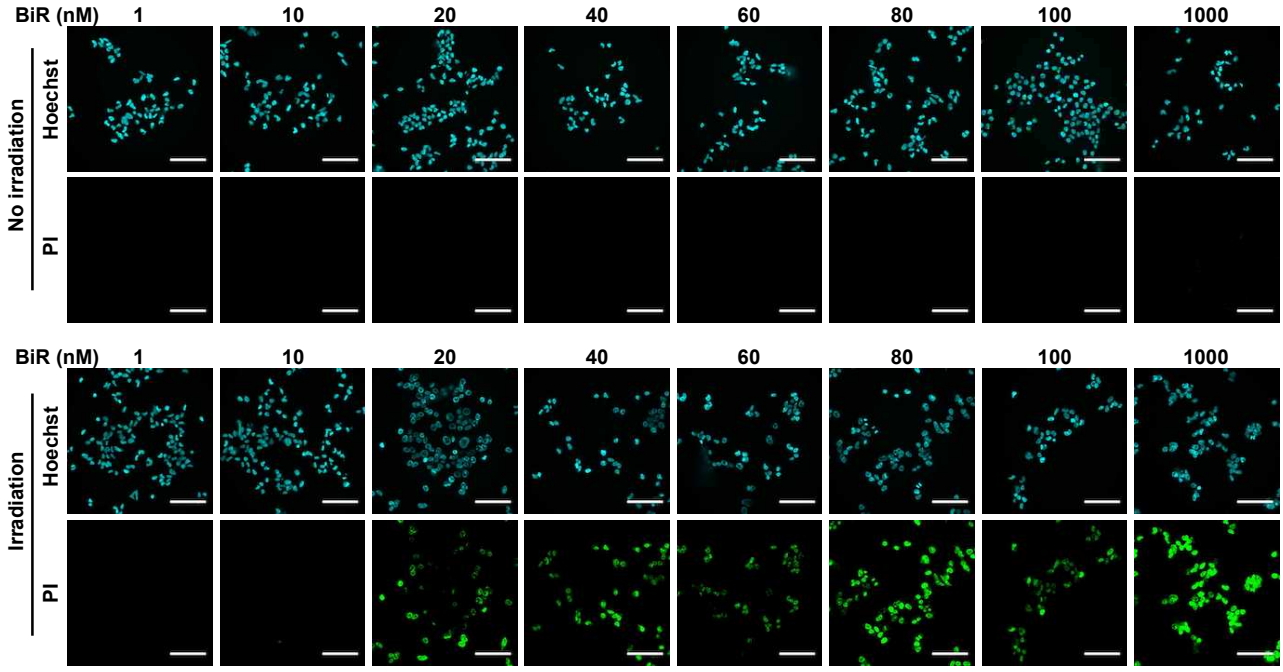


Fig. S6 Fluorescence images of HEK293 cells stained with PI and Hoechst without irradiation (upper) and with irradiation for 1 min in the presence of various concentration of BiR. Scale bars indicate 100 μm.

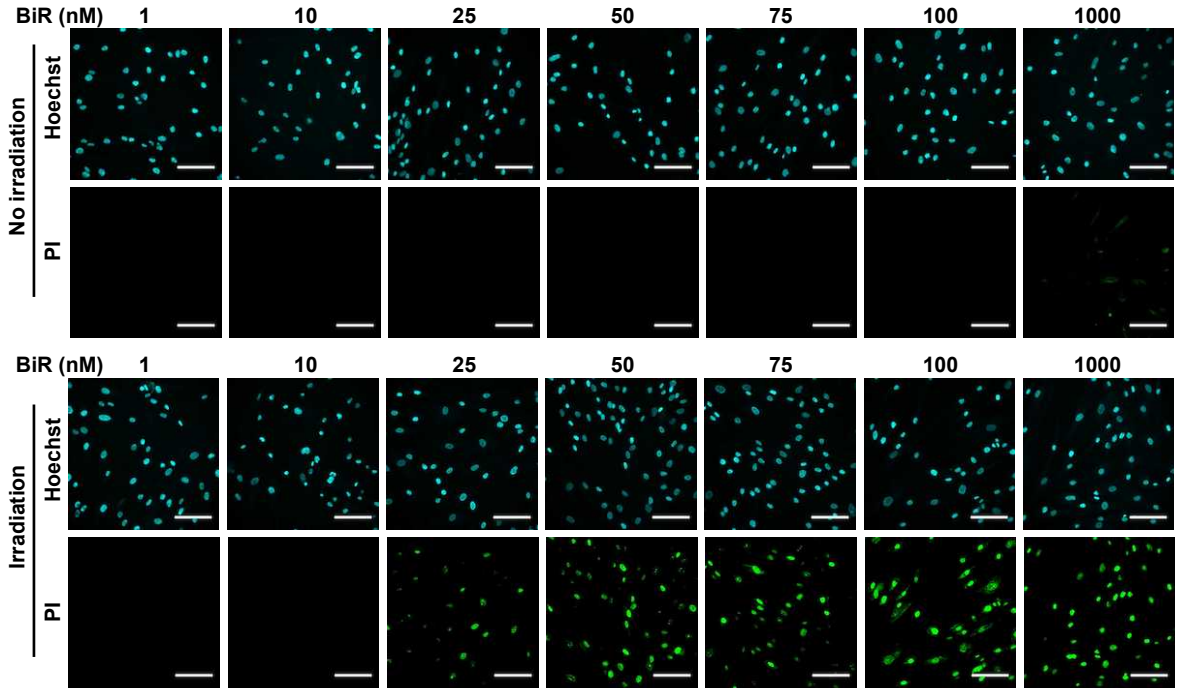


Fig. S7 Fluorescence images of TIG-3 cells stained with PI and Hoechst without irradiation (upper) and with irradiation for 1 min in the presence of various concentration of BiR. Scale bars indicate 100 μm.

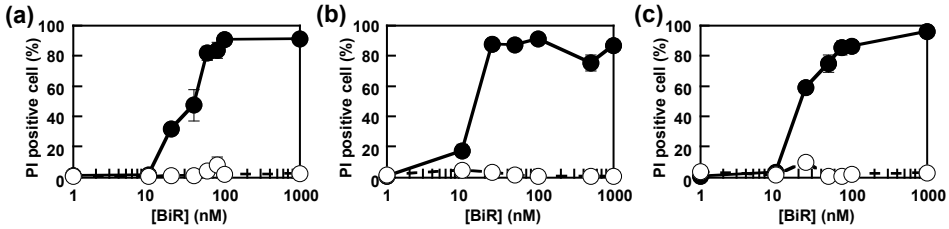


Fig. S8 Plots of the proportion of PI-stained cells to Hoechst-stained cells against concentration of BiR. Filled circles: cells after irradiation. Open circles: cells incubated under dark. (a) A549 cells, (b) HEK293 cells, and (c) TIG-3 cells.

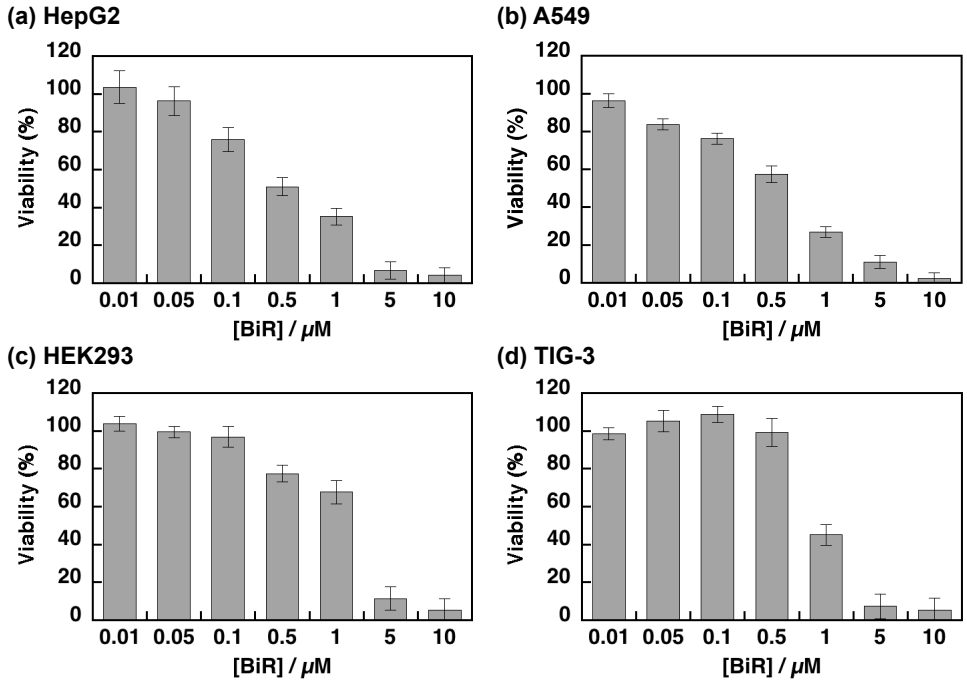
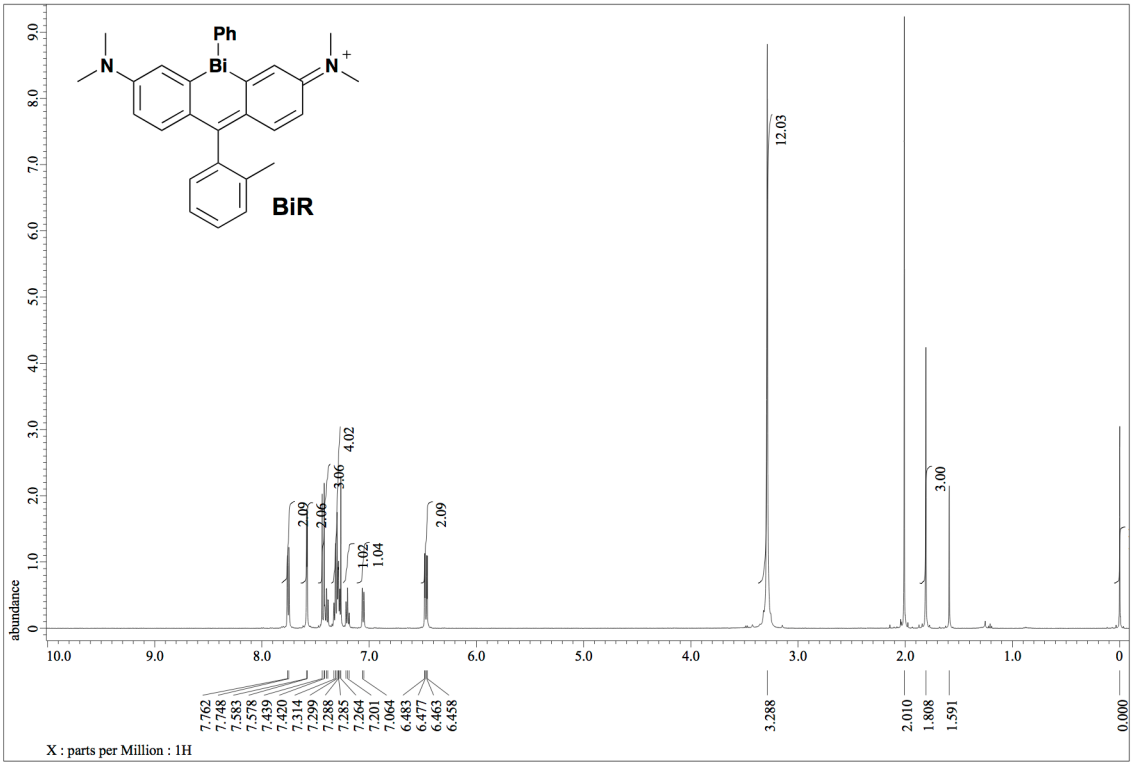
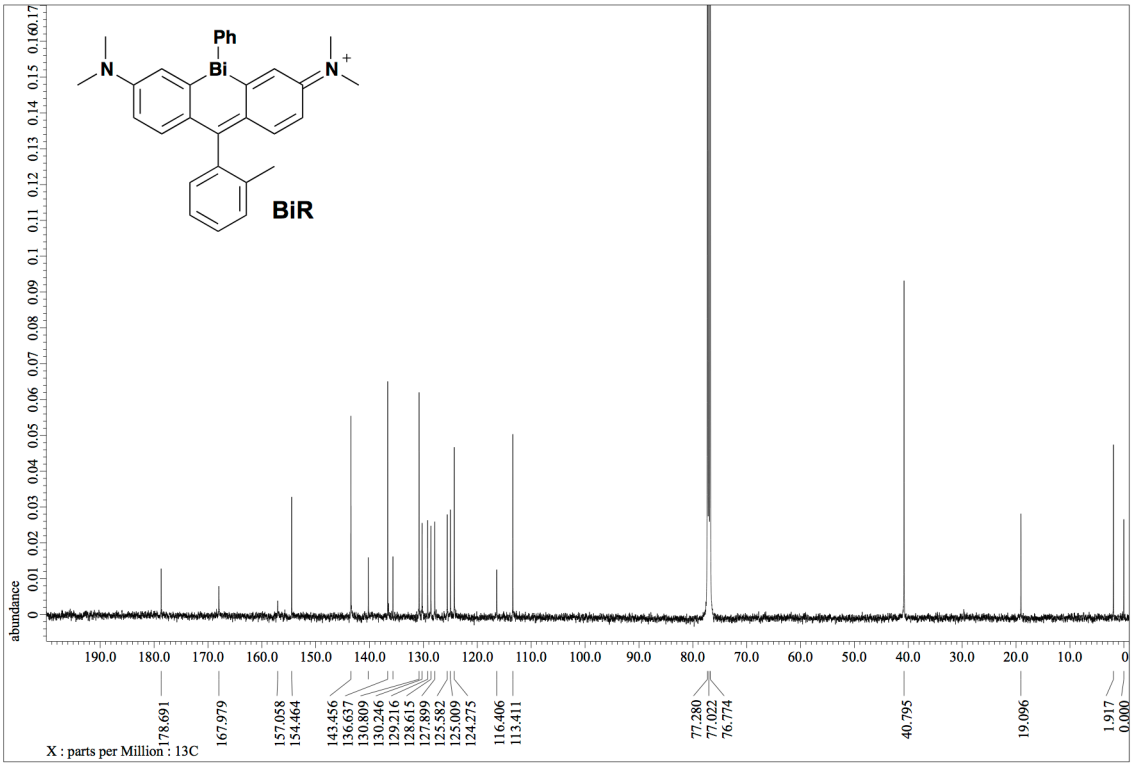


Fig. S9 Cell viability assay (MTT assay) of (a) HepG2 cells, (b) A549 cells, (c) HEK293 cells, and (d) TIG-3 cells after treatment with various concentrations of BiR for 24 h. Error bars indicate standard deviations (n = 3). IC_{50} values were calculated to be 0.53 μM (HepG2), 0.62 μM (A549), 2.26 μM (HEK293), and 0.96 μM (TIG-3).





^{1}H and ^{13}C NMR charts of BiR