Electronic Supplementary Material (ESI) for Chemical Communications.

SUPPORTING INFORMATION

Supplementary Information

A facile two-photon fluorescent probe: Endoplasmic Reticulum tracker, monitoring ER stress and vesicular transport to lysosomes

Pratibha kumari,[a] Sanjay K. Verma[b] and Shaikh M. Mobin,*[a,b,c]

[a]Discipline of Biosciences and Bio-Medical Engineering, [b]Discipline of Chemistry, [c]Discipline of Metallurgical Engineering and Material Science, Indian Institute of Technology Indore, Simrol, Indore 453552, India.

Email: xray@iiti.ac.in

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Experimental Section

Materials and general instruments

2-hydroxy-1-naphthaldehyde and 4-(Aminoethyl)-2-methoxyphenol hydrochloride purchased from TCI Chemicals (India) Pvt. Ltd., LysoTracker Red DND-99 and MitoTracker Red CMXRos, ER-Tracker Red (BODIPY TR Glibenclamide) were purchased from Invitrogen. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tunicamycin and Dithiothreitol (DTT) purchase from Sigma Aldrich Chemical Co., USA. Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco. HeLa cells (cervical cancer cell line), A375 (skin melanoma), MCF-7 (breast cancer) and DU145 (prostate cancer) cells were obtained from National Centre for Cell Science, Pune. All other chemical reagents and solvents were obtained from Merck and S.D Fine Chem.Ltd. Ultrapure water obtains from arium® pro ultrapure water systems (Sartorius) was used throughout the experiment. All commercially available materials were used without further purification.

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured by Bruker Avance (III) instrument by using DMSO-*d6*. Mass spectrum was recorded by Brucker-Daltonics, micrOTOF-QII mass spectrometer. FTIR spectrum was recorded by using Bio-Rad FTS 3000MX instrument on KBr pellets. Spectrophotometric measurements were performed by UV-vis spectrophotometer (Varian Cary 100) using a quartz cuvette with a path length of 1 cm. FluoroMax-4 Spectrophotometer (HORIBA Scientific) was used for Fluorescence measurement. The excitation and emission slits were 5/5 nm for the emission measurements. pH-meter (LABMAN Scientific Instrument PVT. LTD) was used for pH measurements. The absorbance for MTT analysis was recorded by a microplate reader (Synergy H1 BioTek microplate reader) at 570 nm. Fluorescence imaging experiments were performed by Olympus laser-scanning microscope, Mai Tai eHP Spectra physics femtosecond (fs) laser having power peak >450 KW, pulse width ≤ 70 fs, tuning range 690-1040 nm, average power >2.5 W, repetition rate 80 MHz ± 1 MHz was used to acquire the two-photon fluorescence imaging and BD LSRFortessa TM Flow cytometry analysis. Image processing was done with the help of Olympus software (FV10-ASW 4.2). Flow cytometry data were analyzed by DB FACSDiva software. pK_a of imine group and the remote phenolic hydroxyl group of **ERLp** is 3.9 and 9.9 respectively, calculated by ACD software.

Synthesis of Endoplasmic reticulum (ER) targeted ERLp

Condensation reaction was performed from 2-hydroxy-1-naphthaldehyde (172.18 mg, 1 mmol) and 4-(Aminomethyl)-2-methoxyphenol hydrochloride (189.64 mg, 1 mmol) in 20 mL of methanol at 25°C with continuously stirring for 3h in single neck 100 ml RBF (To get the primary amine group free of 4-(Aminomethyl)-2-methoxyphenol hydrochloride, we have treated it with aqueous potassium carbonate). Reaction was monitored by TLC. After completion of the reaction, the solvent was evaporated from rotatory evaporator. The solid product was washed with acidic chilled water and then chilled water only, for several times. The solid orange precipitate was formed, which was filtered and dried in a desiccator. Yield 92%, ^{1}H NMR (400 MHz,DMSO-d_6): δ 14.26 (s, 1H), 9.24 (s, 1H) (d, 1H, -CH=N), 9.01 (s, 1H), 8.09 -8.07 (d, 1H, *ph*), 7.72-7.70 (d, 1H, *ph*), 7.63-7.62 (d, 1H, *ph*), 7.43 (t, 1H, *ph*), 7.19 (t, 1H, *ph*), 7.01 (s, 1H, *ph)*, 6.81- 6.78 (dd, 2H, *ph*), 6.71- 6.68 (d, 1H, *ph*), 4.73(s, 2H), 3.76 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ176.65, 158.31, 147.28, 145.80, 136.62, 133.84, 128.44, 127.89, 127.45, 124.93, 124.80, 121.76, 120.05, 118.07, 115.16, 111.87, 105.35, 55.21, 54.00. FTIR (KBr, cm-1): 3465 (br), 3052 (s), 3006 (s), 2966 (s), 1636 (vs), 1614 (s), 1543 (s), 1453 (s), 1351 (s), 1250 (vs), 1148 (s), 1120 (s), 1065 (vs), 886 (s), 806 (vs), 763 (vs), 647 (s), 587 (s). LCMS (m/z): $[M+H]^+$ 308.1.

1HNMR spectrum of the ERLp after 2 days in DMSO-d_6 solution at 25°C. ^{1}H NMR (400 MHz,DMSO-d_6): δ 14.25 (s, 1H), 9.24 (s, 1H) (d, 1H, -CH=N), 9.01 (s, 1H), 8.09 -8.07 (d, 1H, *ph*), 7.72-7.70 (d, 1H, *ph*), 7.63-7.62 (d, 1H, *ph*), 7.43 (t, 1H, *ph*), 7.19 (t, 1H, *ph*), 7.01 (s, 1H, *ph)*, 6.81- 6.78 (dd, 2H, *ph*), 6.71- 6.68 (d, 1H, *ph*), 4.73(s, 2H), 3.76 (s, 3H).

X-ray crystallography:

The crystal data of **ERLp** was collected at 298 K using graphite-monochromated Mo Kα (λα = 1.54184 Å). The strategy for the data collection was evaluated with the help of CrysAlisPro CCD software. The data were collected by the standard phi-omega scan techniques and were scaled and reduced using CrysAlisPro RED software. The structures were solved by the direct methods by using SHELXS-2014 and refined by full matrix least squares with SHELXL-2014, refining on F^2.[1] By direct methods the positions of all the atoms were obtained and all non-hydrogen atoms were refined anisotropically. All the remaining hydrogen atoms were placed in geometrically constrained positions and refined with isotropic temperature factors, generally 1.2 × Ueq of their

parent atoms. The Hydrogen bonding interactions, mean plane analysis, and molecular drawings were obtained using the program Mercury (ver 3.1) and Diamond (ver 3.1d).[2] The crystal and refinement data are summarized in Table S1 and the selected bond distances and bond angles are shown in Table S2 (Supplementary Information). All the Hydrogen atoms have been omitted for clarity in the molecular structure of **ERLp**.

Two-photon intensity measurement

Mai Tai eHP Spectra physics femtosecond laser having power peak >450 KW, average power >2.5 W, tuning range 690–1040 nm, pulse width ≤ 70 fs, repetition rate 80 MHz ± 1 MHz was used to capture the two-photon fluorescence imaging. The two-photon fluorescence intensity was measured at 700–820 nm. Cervical cancer (HeLa) cells were seeded in confocal dishes and incubated for 24 hours. Live cells were stained with **ERLp** (80 μM) in cell culture medium for 10 min then washed thrice with PBS. Images were captured at a different wavelength. Maximum integrated fluorescence intensity was observed at wavelength 740 nm.

Quantum yield calculation:

The fluorescence quantum yield (ΦF) of **ERLp** was calculated using equation (1) by the steady-state comparative method using tryptophan as the standard (Φ_{st} = 0.14)[3] since excitation wavelength of tryptophan and **ERLp** is similar.

$\Phi_x = \Phi_{st} \times S_x/S_{st} \times A_{st}/A_x \times \eta^2_x / \eta^2_{st}$ equation (1)

where St and X denote standard and unknown respectively. Φ_x is the fluorescence quantum yield of the unknoun sample (test sample), Φ_{st} is the fluorescence quantum yield of the standard sample, S_x and S_{st} are the integrated emission band areas of the unknown sample and the standard respectively. A_{st} and A_x represent the absorbance of the standard and the unknown sample at the excitation wavelength, respectively. η^2_x and η^2_{st} are the refractive index of the solvent of test sample and the standard sample. Here both standard sample tryptophan and **ERLp** dissolve in water. Therefore refractive index of solvent is same in both case.
The fluorescence quantum yield (ΦF) of ER traker red was calculated using above equation (1). In this case quinine sulfate used as the standard (Φst = 0.54)[4].

MTT cell viability assay [5]

Cell viability assay of **ERLp** was performed on Hela cells, 6000 HeLa cells well^{-1} were seeded in 96-well plate in 200 µL media (Dulbecco's Modified Eagle Medium, 10% (v/v) FBS and 1% Penicillin-Streptomycin antibiotics 10,000 U/mL) and incubated for one day for cell adhesion. Next day media was removed and fresh media 100 µL added in each well. A stock solution of **ERLp** was prepared in 0.05% acetonitrile and rest water. 20 µL added to each well in order to give final concentration ranging from 10-140 µM. 20 µL water having 0.05% acetonitrile was used as a control. The experiment was done in triplicate. Cells exposed to the drug for 24 hours followed by washing with PBS (pH 7.4) and 100 µL MTT (1 mg/mL in phenol red free media) was added in each well. Cells incubated for next 4 hours at 37 °C and 5% CO_2 atmosphere. After that media was carefully removed and 100 µL DMSO was added in order to dissolve purple formazan crystals. After 15 minutes incubation, absorbance at 570 nm was measured using Synergy H1 Biotek microplate reader. The % cell viability was calculated as: % cell viability = [Mean O.D. of the drug-treated cell/Mean O.D. of the control well] × 100

Flow cytometry

HeLa cells were cultured in six-well tissue culture plates. When cells became 80% confluent then one well treated with 40 µM probe **ERLp** and another well with 80 µM **ERLp** for 10 min and control well left without any treatments. After that, the cells were washed with PBS three times. Then harvested by trypsin and re-suspended in PBS. The samples were analyzed by BD LSRFortessa TM Flow cytometry with excitation at 405 nm and emission 415–470 nm. Data were analyzed by DB FACSDiva software. Total ten thousand events were acquired for each sample.

Concentration dependent confocal imaging of ERLp

HeLa cells were seeded in confocal dishes. Cells in each dishes treated with diffrent concentration of **ERLp** (5µM, 20 µM, 40 µM, 60 µM, 80 µM) for 10 min followed by washing with PBS. In other case incubation time of **ERLp** increased to 2 h by keeping concentration

constant (5μM, 20 μM, 40 μM, 60 μM, 80 μM). After 2 h washed with PBS and images captured by confocal microscope.

Endoplasmic reticulum (ER) staining and co-localization studies in various cell lines

Cervical cancer (HeLa), skin melanoma (A375), prostate cancer (DU145) and Breast cancer (MCF-7) cell lines were seeded in confocal dishes and incubated for 24 hours for cell attachment with the bottom surface of dishes. For ER imaging, live cells were stained with **ERLp** (80 μM) in cell culture medium for 10 min then washed thrice with PBS (pH = 7.4) and co-stain with ER tracker Red (1μM, 30 min), LysoTracker Red DND 99 (80nM, 30 min), MitoTracker Red (90 nM,15 min) for ER, lysosomes and mitochondria respectively in separate dishes. Cells incubated at 37°C in 5% CO_2 atmosphere. An Olympus laser scanning microscope was used for confocal imaging. Cells ware visualized at an excitation of λ_{ex} = 405 nm for **ERLp** in case of one photon excitation and λ_{ex} = 740 for two-photon excitation, λ_{ex} = 559 nm for ER tracker red, Lyso tracker red as well as Mito tracker red.

Cell uptake pathway studies

In order to study the probable mechanistic pathway of cellular uptake of **ERLp**, HeLa cells were seeded in two confocal dishes and incubated at 37°C for 24h. The cells seeded in two separate dishes were exposed with **ERLp** (80 μM) at 37°C or 4°C for 1 h. After that cells were washed with PBS three times and images captured by a confocal microscope at excitation 405 nm and emission signal collected at 415-470 nm.

Photostability study of ERLp

Photostability is an important factor that play significant role in fluorescent imaging purpose. Photobleaching experiment was performed, both in solution as well as on live cells to check the photostability of **ERLp**. Solution of **ERLp** in water was continuously UV illuminated (254 nm) at 25 °C for upto 80 min, fluorescence intensity of **ERLp** measure after every 10 min upto 80 min by FluoroMax-4 Spectrophotometer (Fig. S20).

To further evaluate whether **ERLp** is also photostable for Endoplasmic Reticulum imaging in living cell. HeLa cells were seeded in confocal dishes and incubated overnight for cell attachment. Dishes were incubated with **ERLp** (80 μM, 10 min). After 10 min incubation, washed

thrice with PBS to remove unbounded probe in media. The cells were imaged using Mai Tai eHP Spectra physics femtosecond laser used for two-photon imaging with no delay scan mode. Videos and images were recorded up to 1800 scans. **ERLp** were excited at 740 nm for two-photon imaging. The maximum intense signal of the first scan was considered 100% for measuring the relative decrease in intensity. Images at every 200 scans were obtained from the video. The relative percent intensity calculated as F/F_0*100.

Prolong ER stress leads to cells apoptosis[6]

HeLa cells in confocal dishes were treated with **ERLp** (80 μM) and incubated for 10 min followed by washing with PBS. The cells were co-stained with ER tracker red (1μM, 30 min) and again washed with PBS. Tunicamycin 40μg/ml (Tunicamycin 1mg/ml stock solution in DMSO) added in 1ml media in the confocal dish. Immediately images were captured after a different time interval to monitor morphological changes in cells due to excess ER stress in real time. Further to explore more ER stress study, Dithiothreitol (DTT) which known as strong reducing agent and blocks protein disulfide- bond formation. So in another experiment cells were treated with **ERLp** and co-treated with ER tracker red followed by washing with PBS buffer. Then 200 μM DTT (solublized in Milli-Q water) added in 1 ml DMEM media and images captured after different time intervals.

ER to lysosome vesicles transports study

In order to study vesicles transport from ER to lysosomes, HeLa cells were seeded in two separate dishes. In both dishes, cells were treated with **ERLp** (80μM, 10 min) and unbonded probe washed with PBS. One dish co-stained with ER tracker red and another dish co-stained with Lyso tracker red followed by washing with PBS. Then images were captured in a different time interval to monitor **ERLp** transported from ER to lysosome. As Pearson's colocalization coefficient decreases in the case of ER tracker red and increases in case of Lyso tracker red.

Generation of multicellular 3D spheroids

Multicellular tumor spheroids (MCTSs) were produced using the liquid overlay method.[7] Flat-bottom 96 well plates were coated with 50µL of a sterile 1.5% (wt/vol) warm agarose solution in complete DMEM media to make a non-adherent surface. After that HeLa cells (~70% confluent in T-25 flack) were harvested by 0.25% trypsin/EDTA solution and re-suspended in DMEM media. Approx 3000 cells were seeded in each agarose coated well in 200µl complete DMEM media. The plates were incubated at 37°C and 5% CO_2 until spheroids formed. For compound treatment and imaging, spheroids were gently transfers in confocal disc with the help of 1ml pipette. Spheroids were treated with 80 µM **ERLp** and incubated for 1h, then washed gently with PBS three to four times and images were captured by two-photon microscopy as well as one photon confocal laser scanning microscopy.

Result and discussions

Crystal packing discription of ERLp

The packing features of **ERLp** reveal the presence of intra molecular H-bonding interaction O(3)–H(3)···N(1) = 1.885(0) Å and inter molecular H-bonding interactions O(3)–H(2)···O(2) = 1.835(0) Å, O(3)–H(3)···O(3) = 2.561(1) Å resulting in a 1D-chain along *c*-axis (Fig. S7). This 1D-chains are further interconnected to each other by H-bonding interactions through C(18)–H(18)···O(2) = 2.606(1) Å forming supramolecular 2D-network along *a*-axis, where C(18)–H(18) is donor and O(2) is acceptor (Fig. S8). It is to be noted that the presence of four kinds of donor-acceptor interactions O(3)–H(2)···O(2) = 1.835(0) Å, C(9)–H(9)···O(2) = 2.661(0) Å, C(9)–H(9)···O(1) = 2.561(0) Å and C(18)–H(18)···O(2) = 2.606(1) Å which provide an opportunity to extend the dimensionality to supramolecular 3D-architecture along *b*-axis (Fig. S9). Further, the 3D supramolecular network is stabilized by π···π stacking interactions (3.931 Å) between the two phenyl rings of the different moieties in a *tail-to-tail* arrangement (Fig. S10).

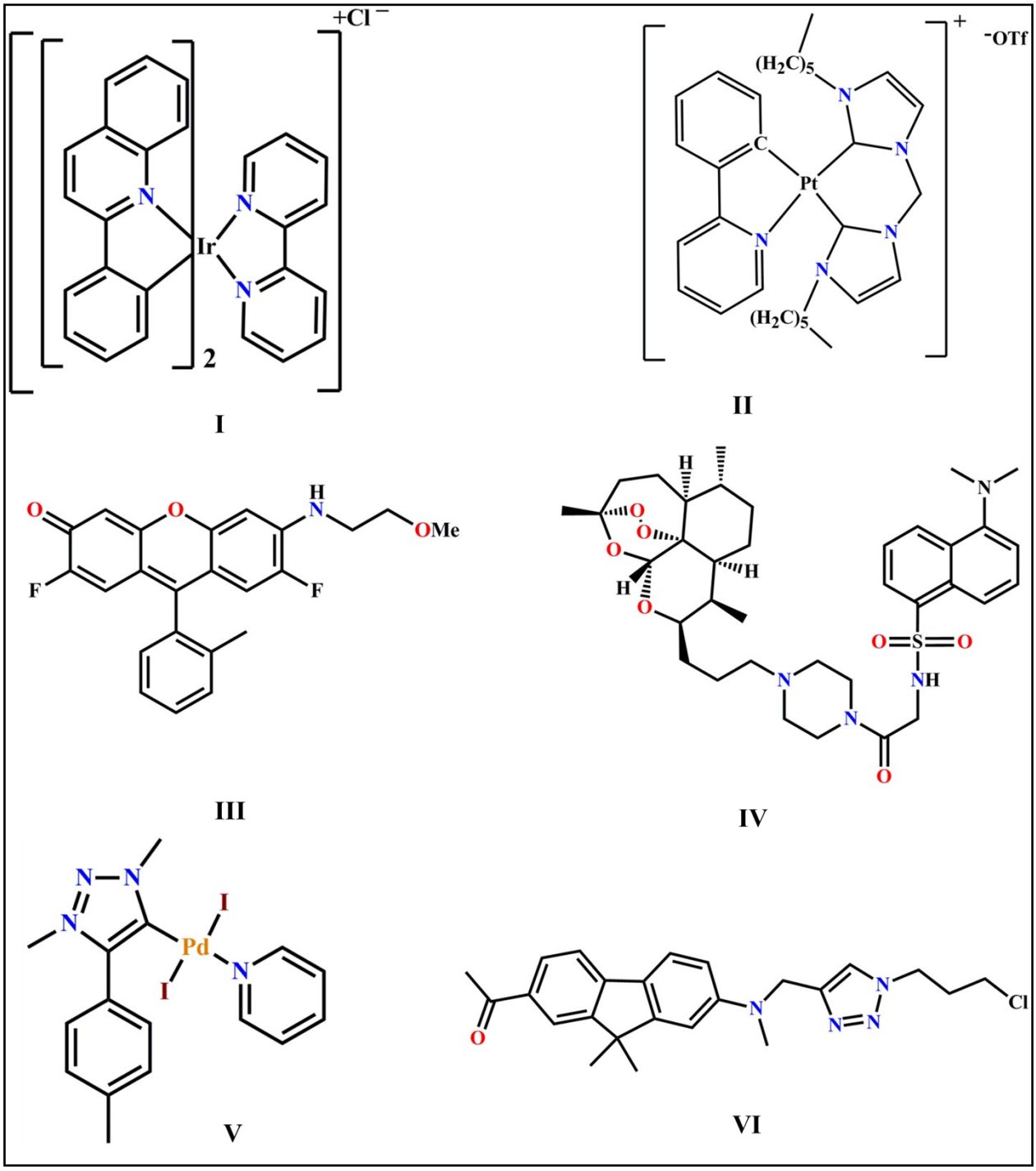

Chart S1. ER targeting multistep reported compounds. [8]

Supporting figures

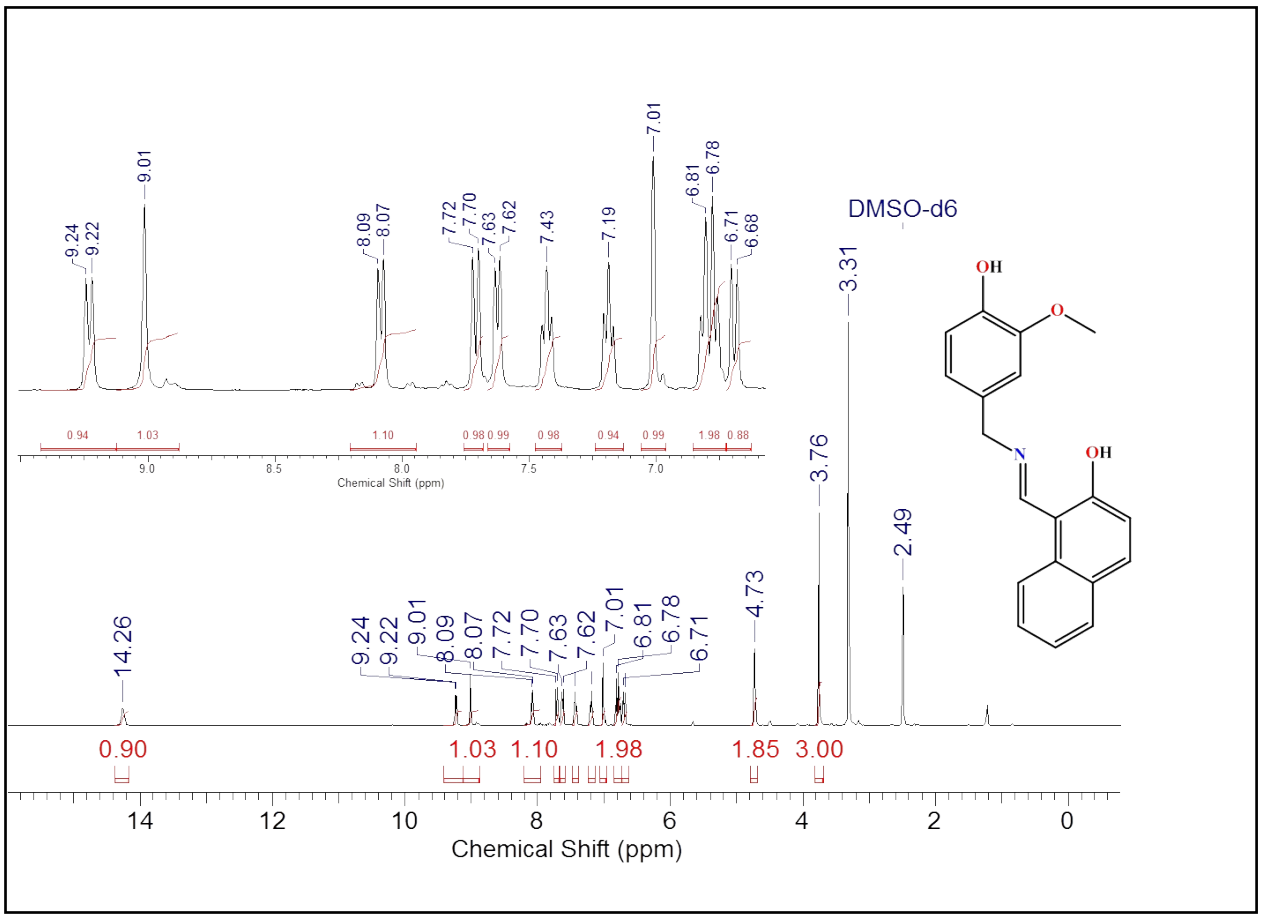

Fig. S1. ^{1}H NMR spectrum of **ERLp**.

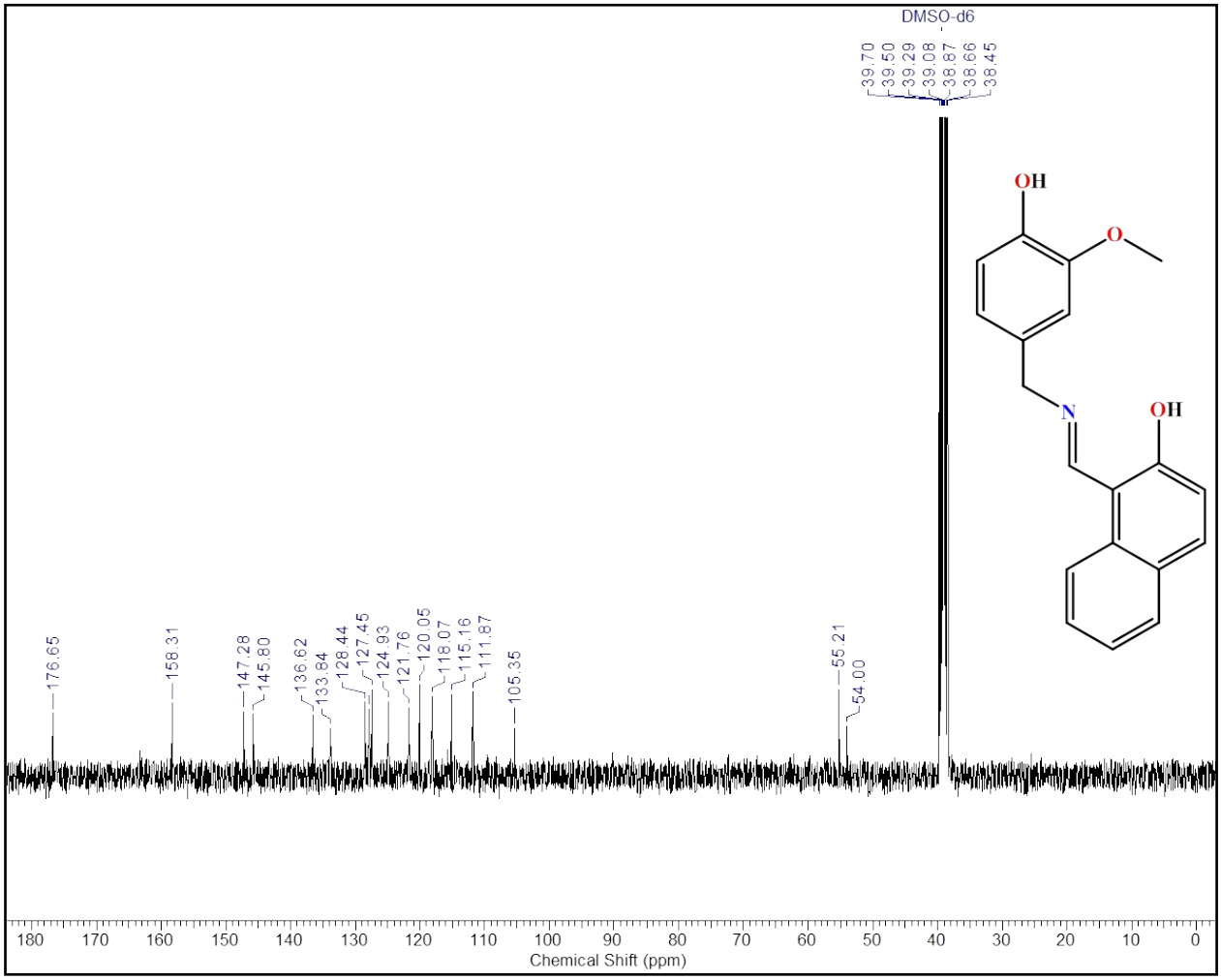

Fig. S2. ^{13}C NMR spectrum of **ERLp**.

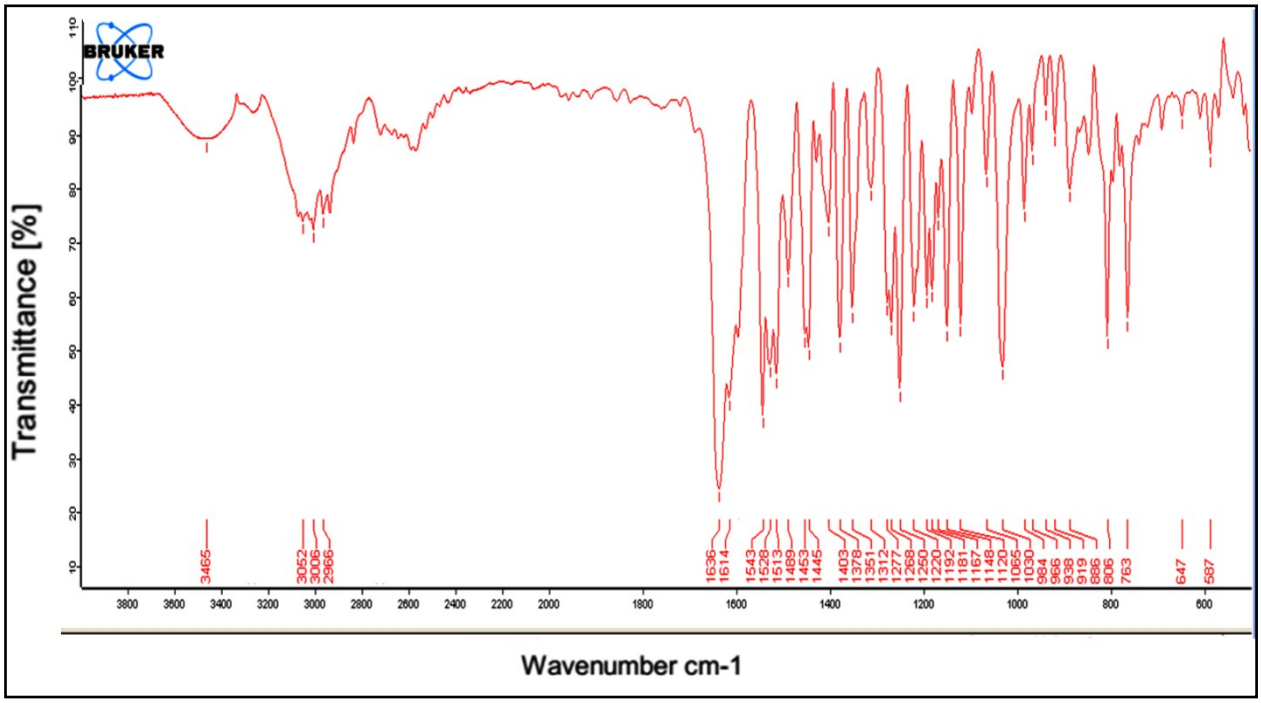

Fig. S3. FTIR spectrum of **ERLp**.

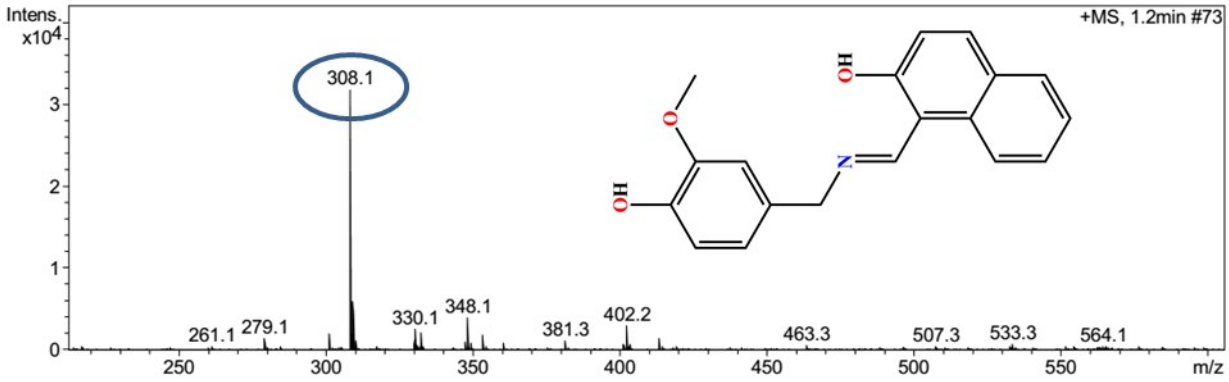

Fig. S4. LCMS spectrum of **ERLp**.

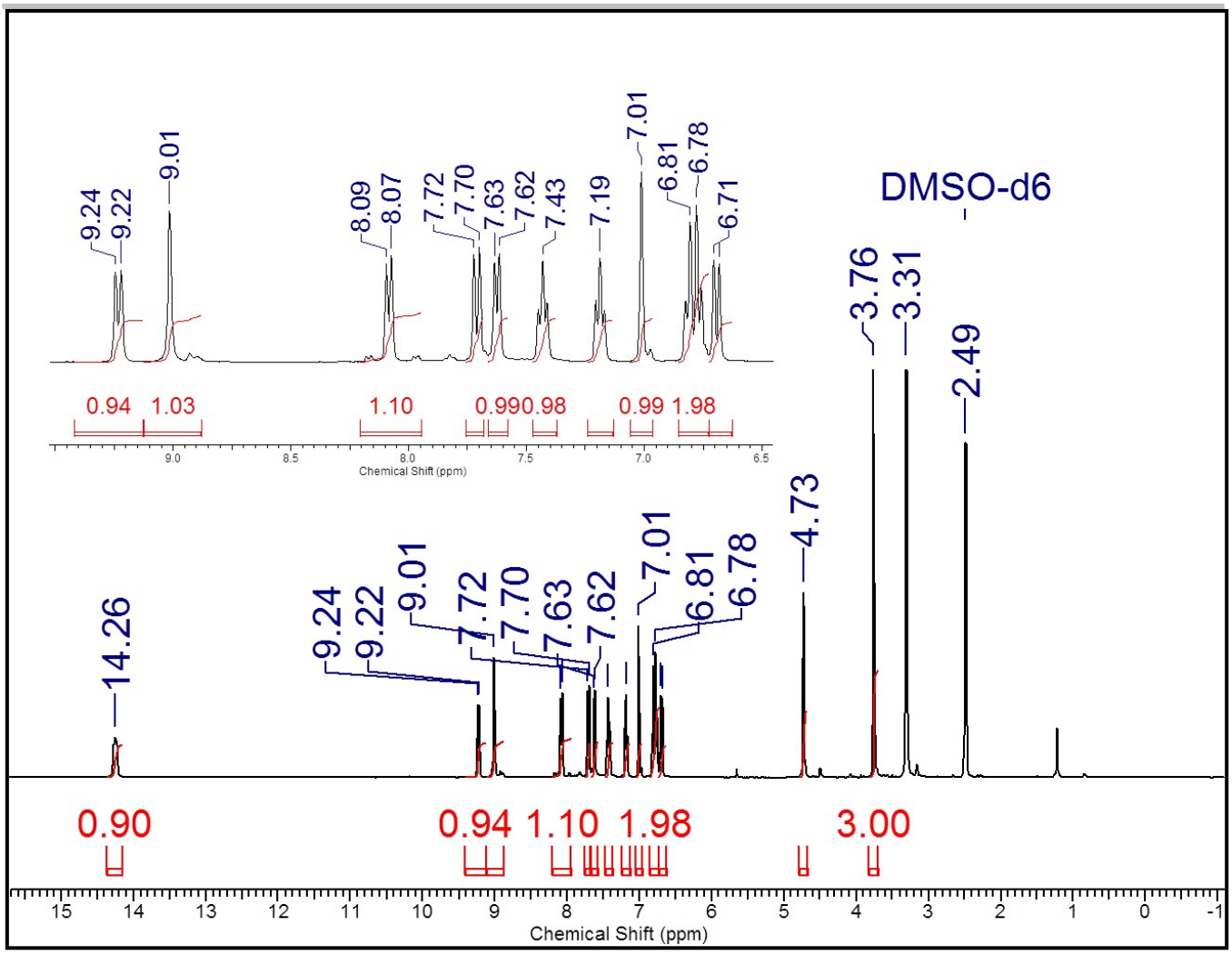

Fig. S5. Chemical stability of **ERLp** was quantified by ^{1}H NMR spectrum**. ERLp** solution in DMSO-*d6,* kept at 25 °C for 24 hours then ^{1}H NMR spectrum measured.

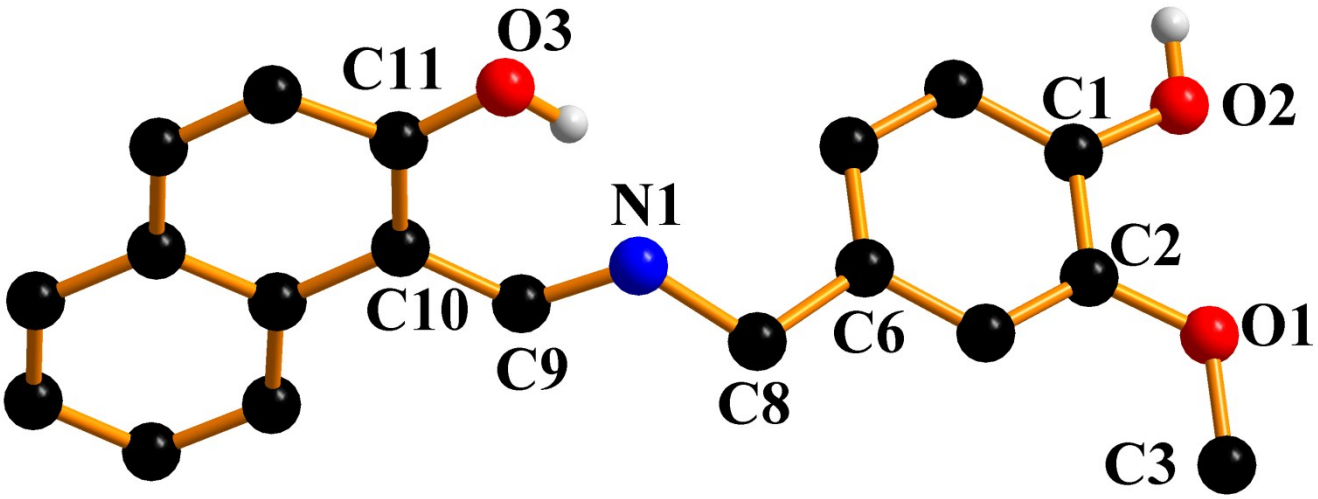

Fig. S6. Crystal structure of **ERLp**

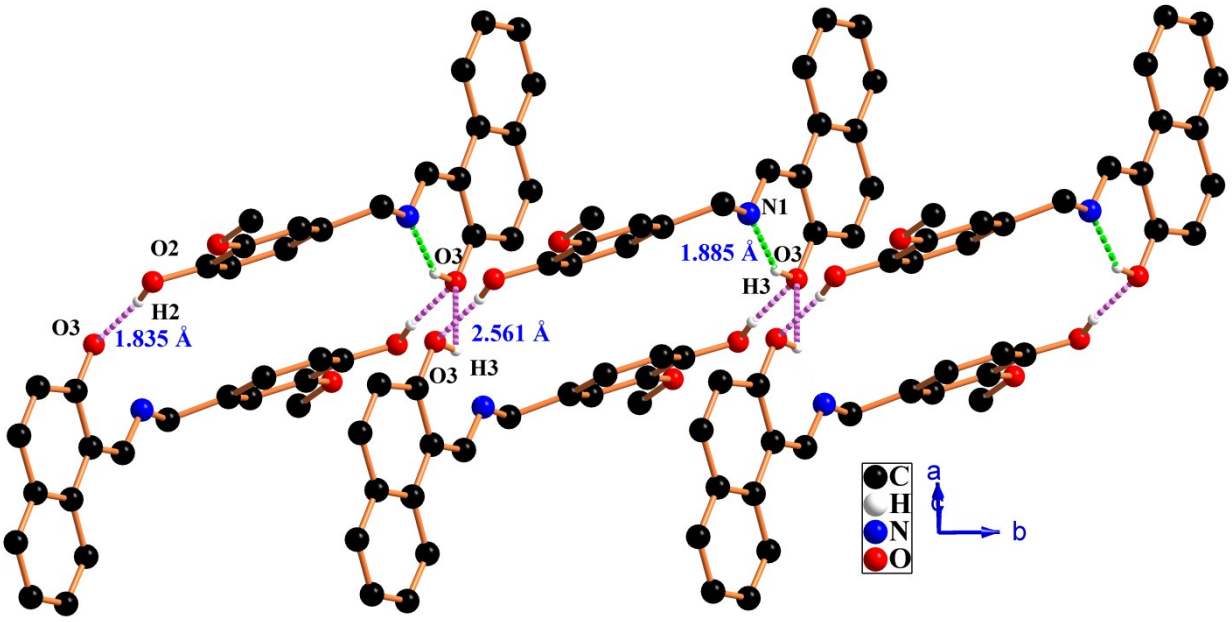

Fig. S7. 1D chain along *c*-axis in **ERLp** in the presence of inter and intra molecular H-bonding (Purple and green dots represent inter and intra H-bond respectively).

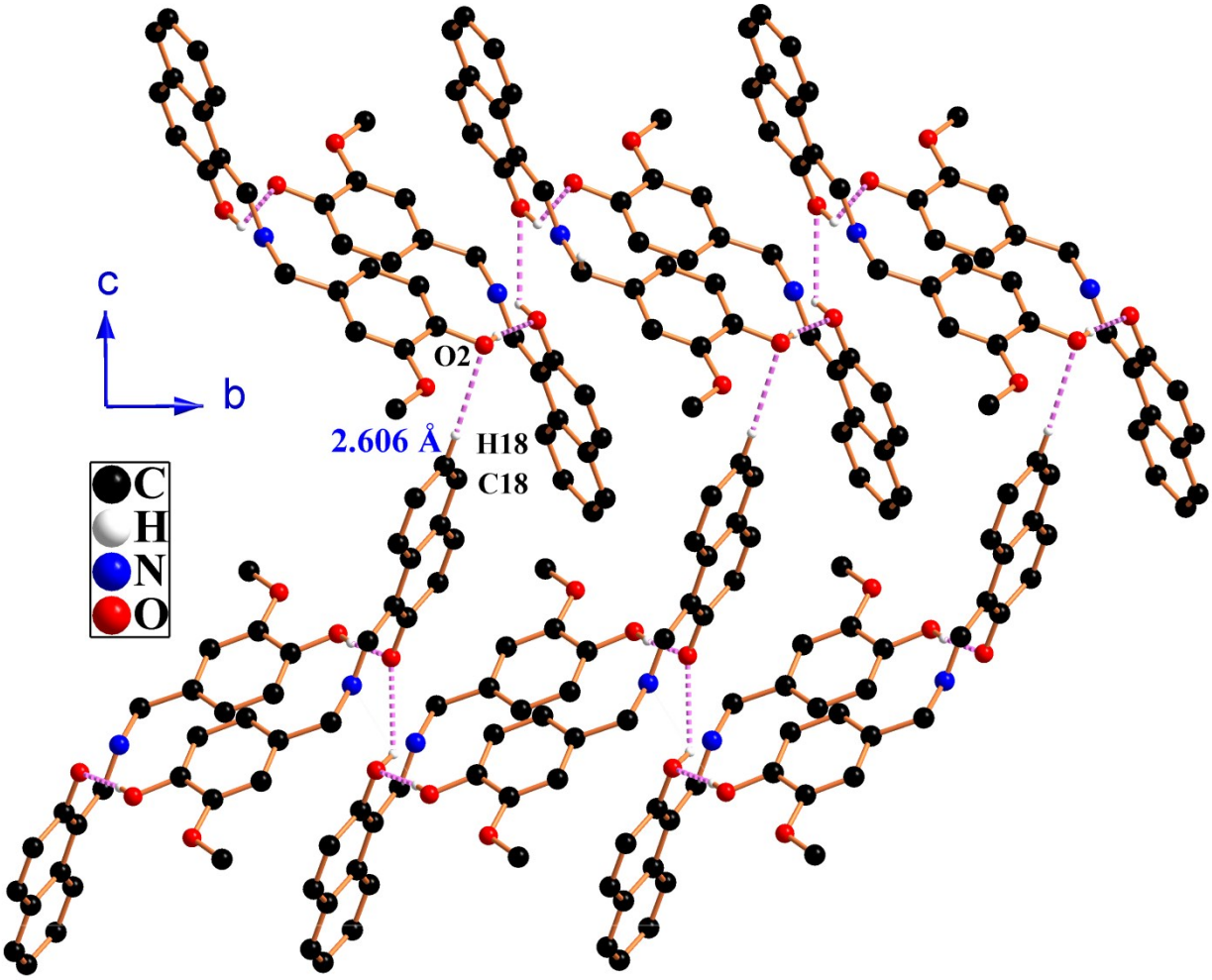

Fig. S8. 2D network chain of **ERLp** along *a*-axis in the presence of inter molecular H-bonding (Purple dot represent H-bond).

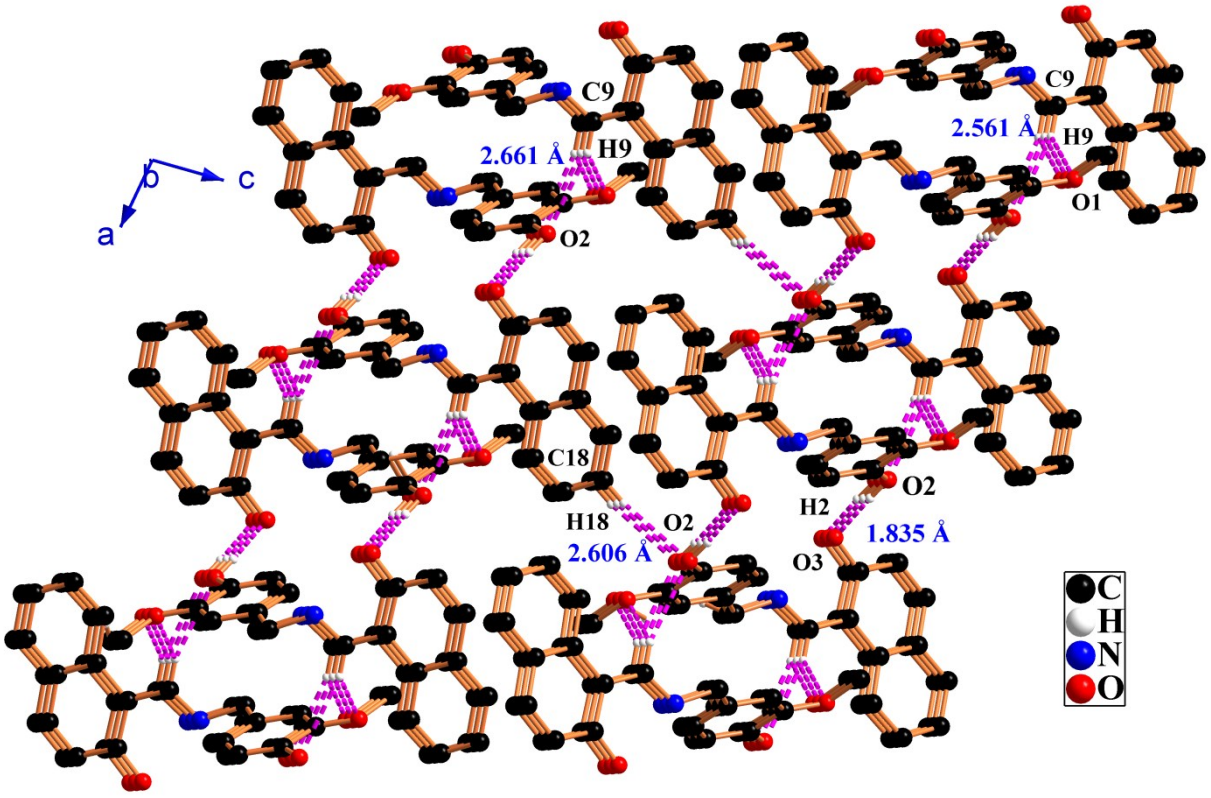

Fig. S9. 3D network of **ERLp** along *b*-axis in the presence of inter molecular H-bonding (Purple dot represent H-bond).

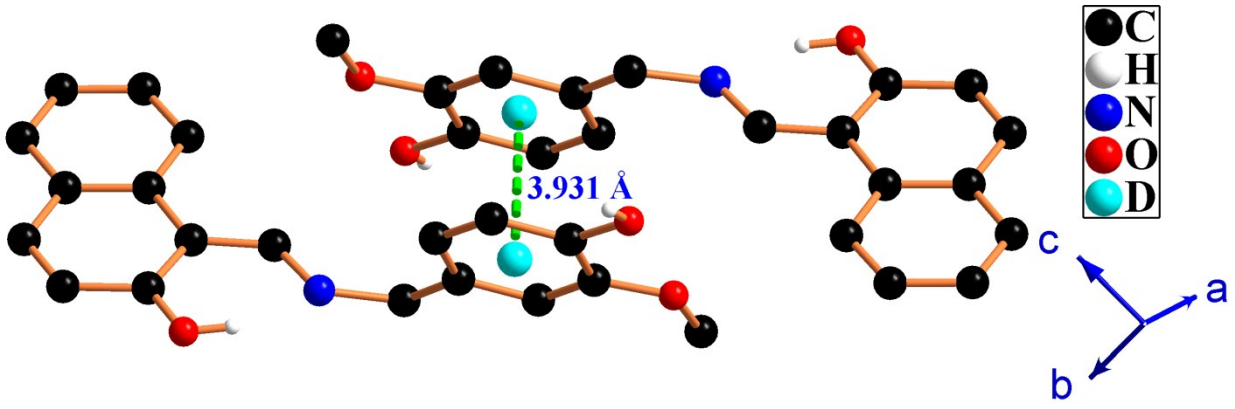

Fig. S10. Ball and stick model of **ERLp** showing the π···π interaction between two phenyl rings of a different molecule. (fragmented green line represented as π···π interaction).

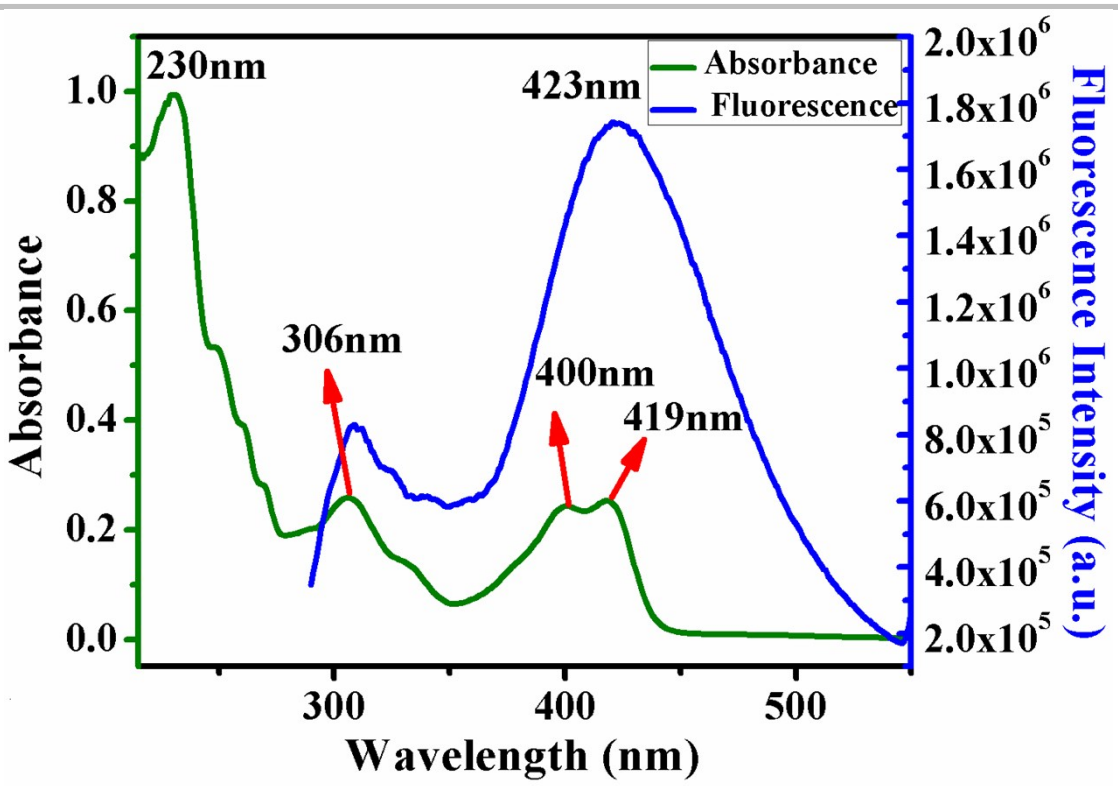

Fig. S11. Absorption and emission spectra of ERLp (10 μM) in acetonitrile solution.

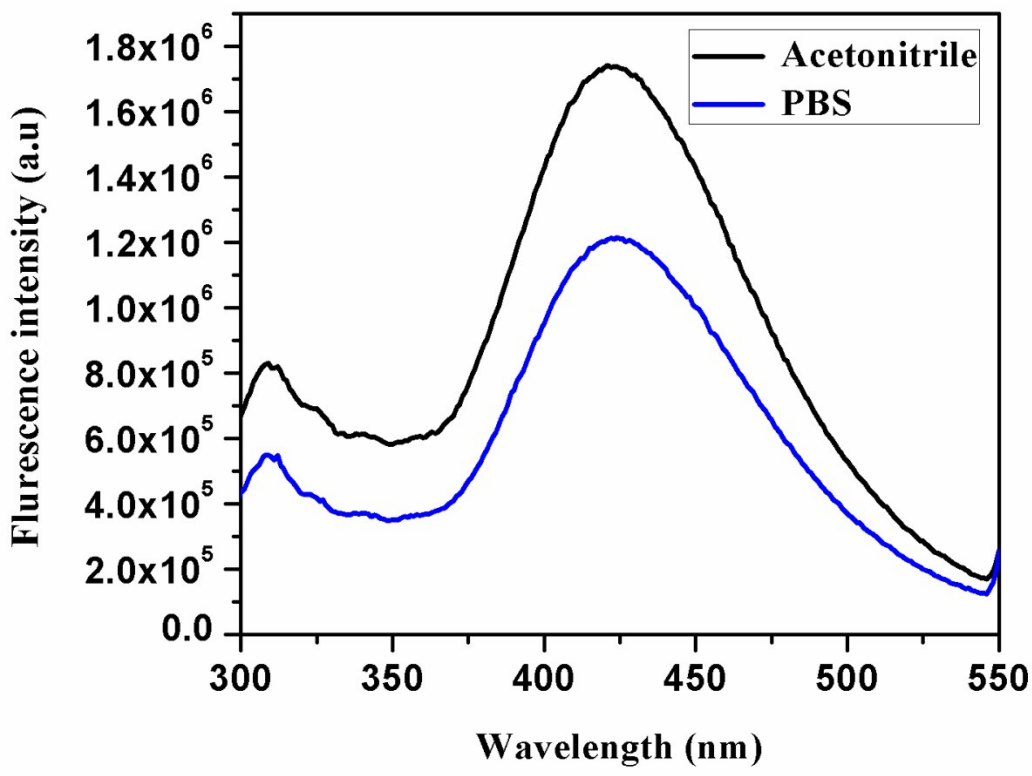

Fig. S12. Emission spectra of **ERLp** (10μM) in acetonitrile and PBS.

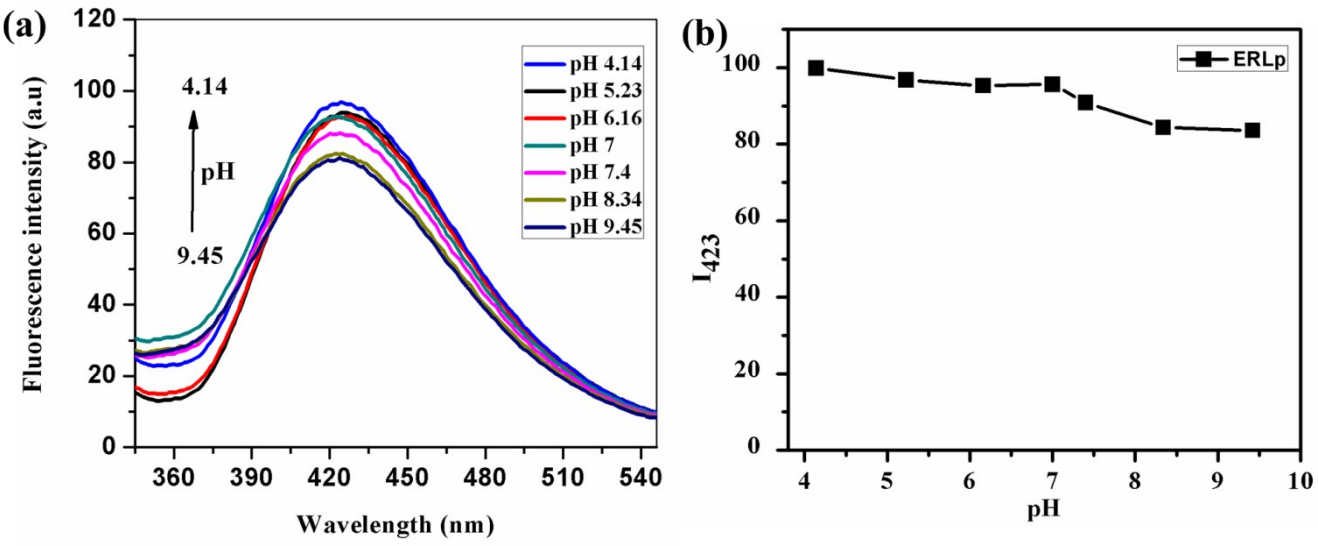

Fig. S13. (a) pH-sensitive emission spectra of **ERLp** (10 μM) in PBS buffer. (b) The plot of the emission intensity of **ERLp** at 423 nm versus various pH values.

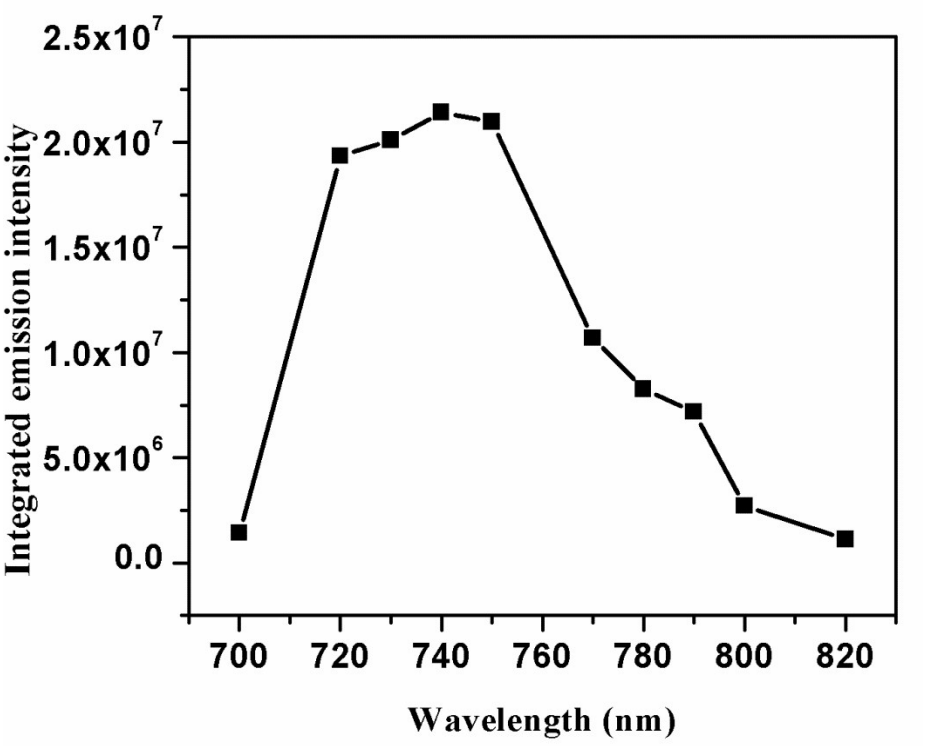

Fig. S14. Two-photon integrated fluorescence emission intensity of **ERLp** at excitation wavelengths 700–820 nm in live HeLa cells.

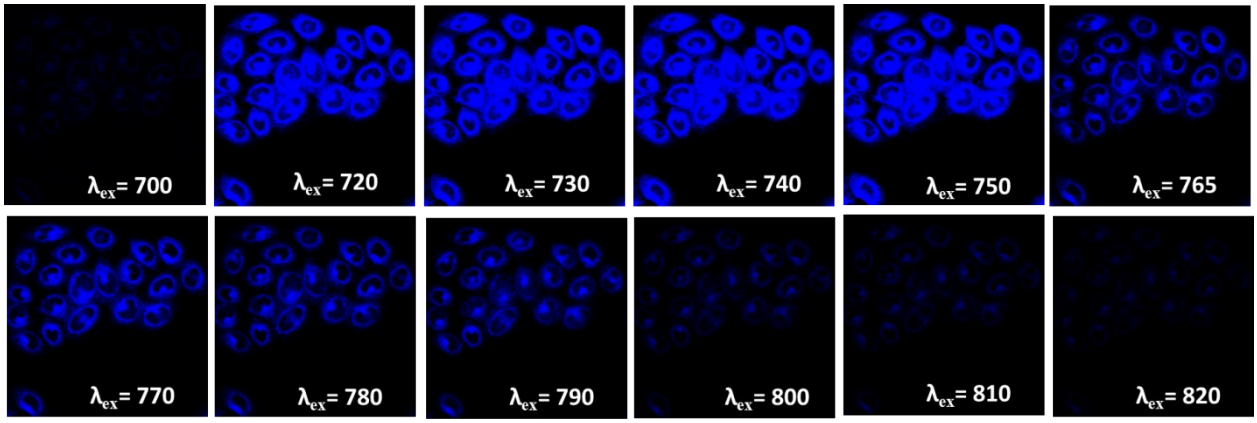

Fig. S15. Two-photon microscopy (TPM) images of HeLa cells labeled with **ERLp**. The excitation wavelength was 700 to 820 nm. Maximum fluorescence intensity was obtained at λ_{ex} = 740 nm

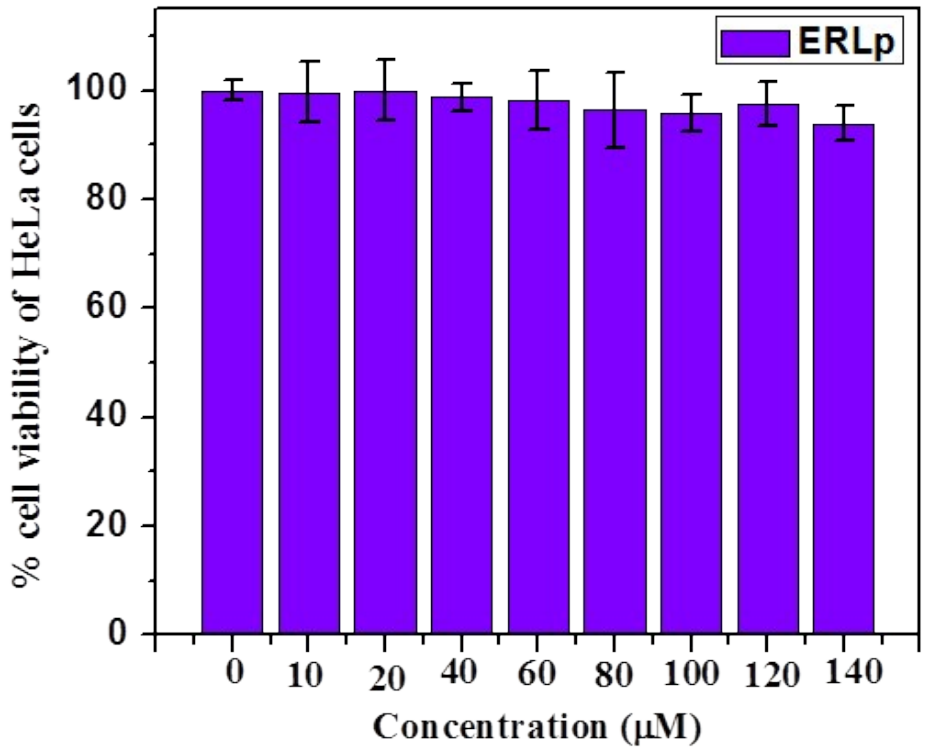

Fig. S16. Cell viability assay of **ERLp** was determined by the MTT assay after 24 h incubation at 37°C and the results shown in mean ± SD of three separate measurements.

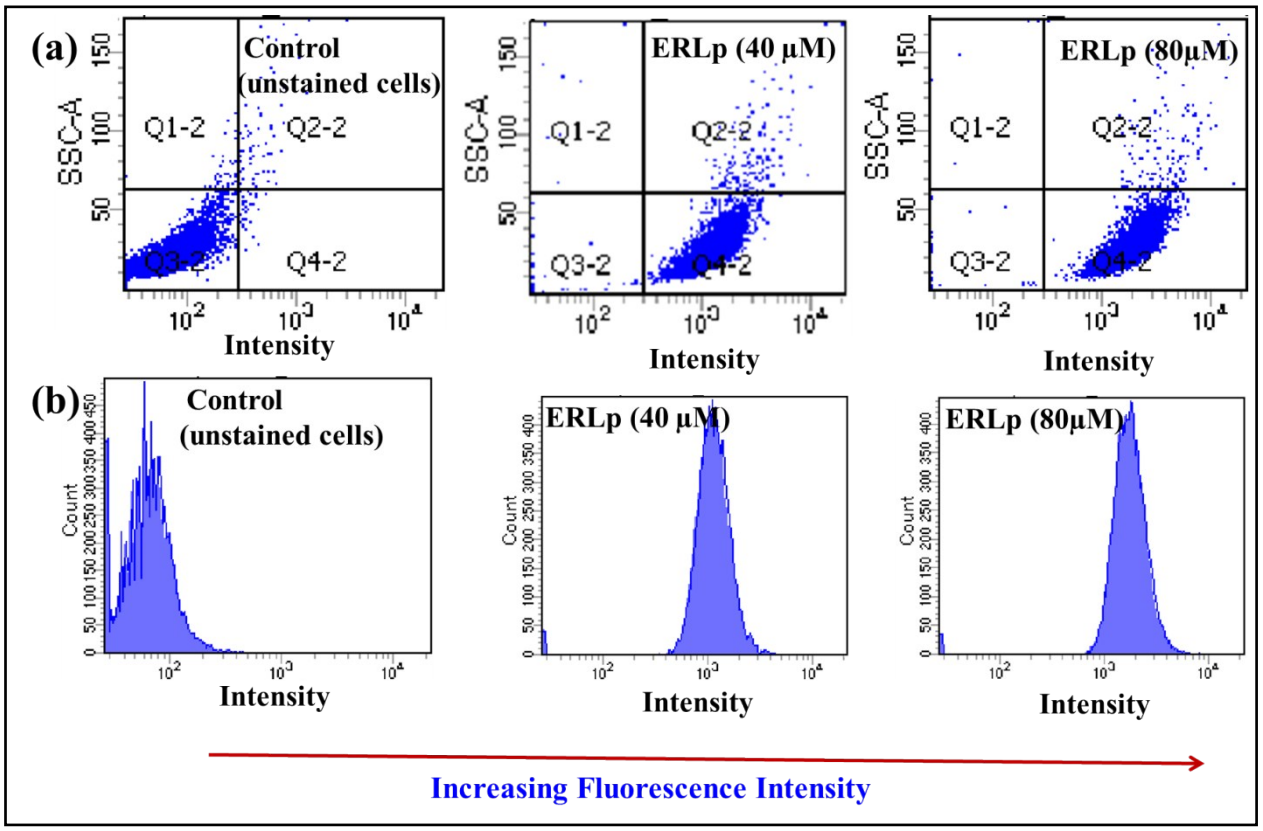

Fig. S17. Concentration-dependent flow cytometry analysis of **ERLp** in live HeLa cells. (a) Scatter plot. (b) Histogram. **ERLp**: λ_{ex} = 405 nm; λ_{em} = 415–470 nm.

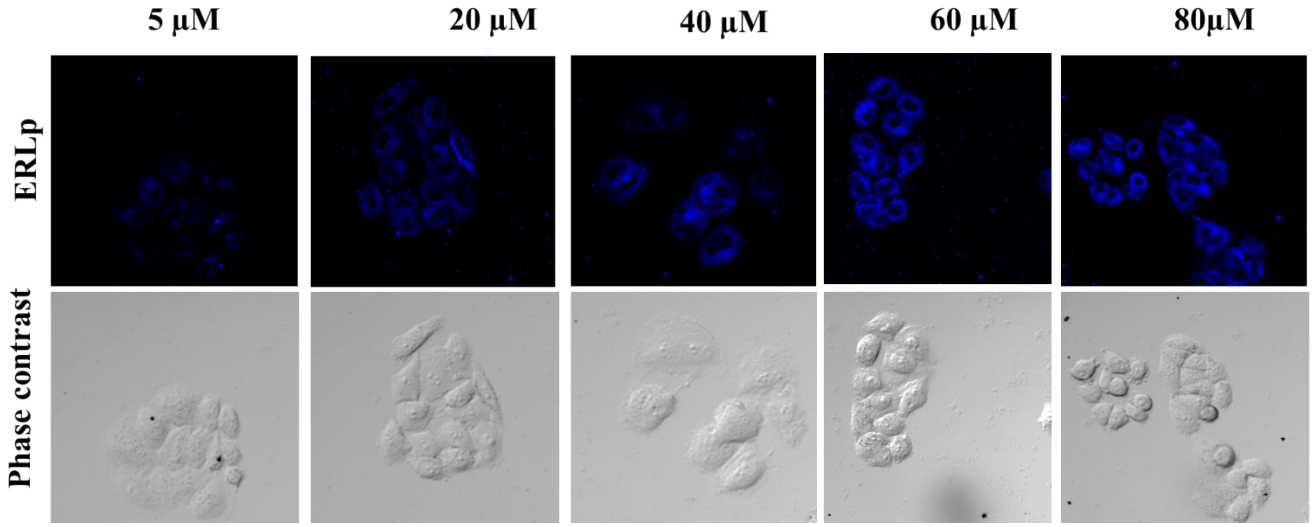

Fig. S18. Concentration-dependent staing of live cells using **ERLp** for 10 min incubation time. (λ_{ex} = 405 nm; λ_{em} = 415-470 nm).

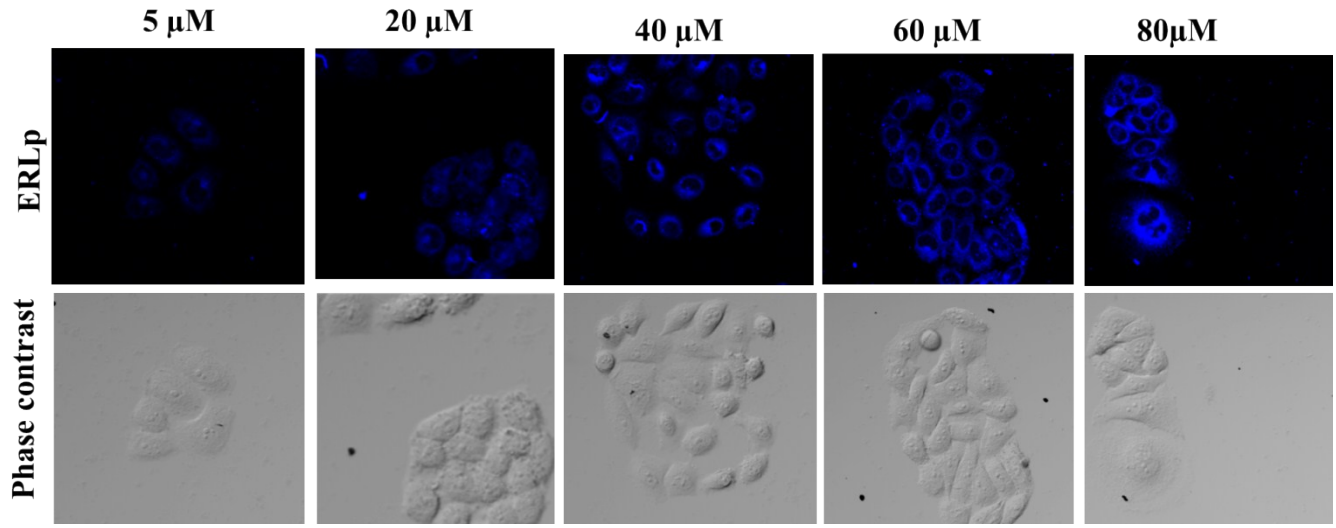

Fig. 19. Concentration-dependent staing of live cells using **ERLp** for 2h incubation time. (λ_{ex} = 405 nm; λ_{em} = 415-470 nm).

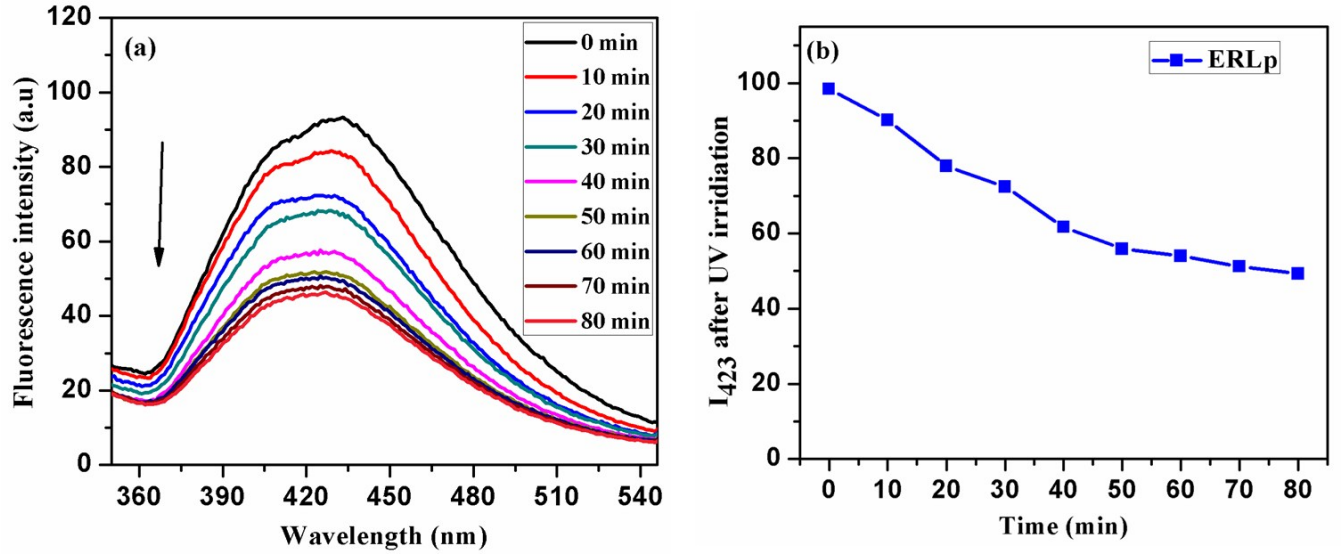

Fig. S20. Photobleaching study of **ERLp** (10 µM) in water under UV illumination (254 nm). (a) Fluorescence intensity measure after different time interval. (b) The plot of the emission intensity of **ERLp** at 423 nm versus exposure time (min) of UV rays.

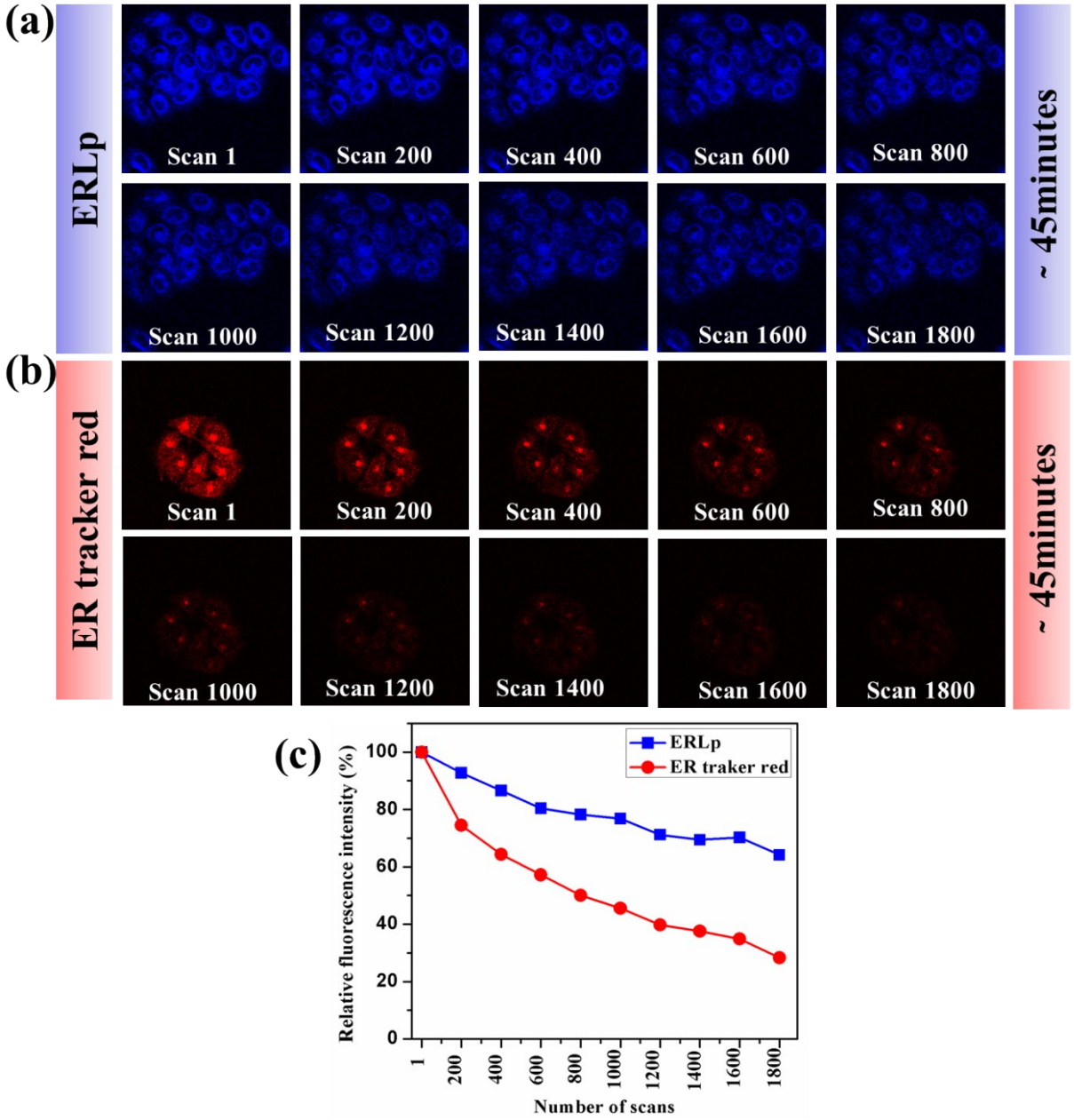

Fig. S21. Comparisons of the *in vitro* photostability of ER-Tracker Red and **ERLp** in HeLa cell lines. (a) **ERLp** (80 μM): λ_{ex} = 740 nm, λ_{em} = 415–470 nm) photobleaching HeLa cells. (a) ER tracker red (1μM): λ_{ex} = 559 nm, λ_{em} = 580–700 nm) photobleaching study in HeLa cells. (b) Graph showing photobleaching result of **ERLp** and ER tracker red**.**

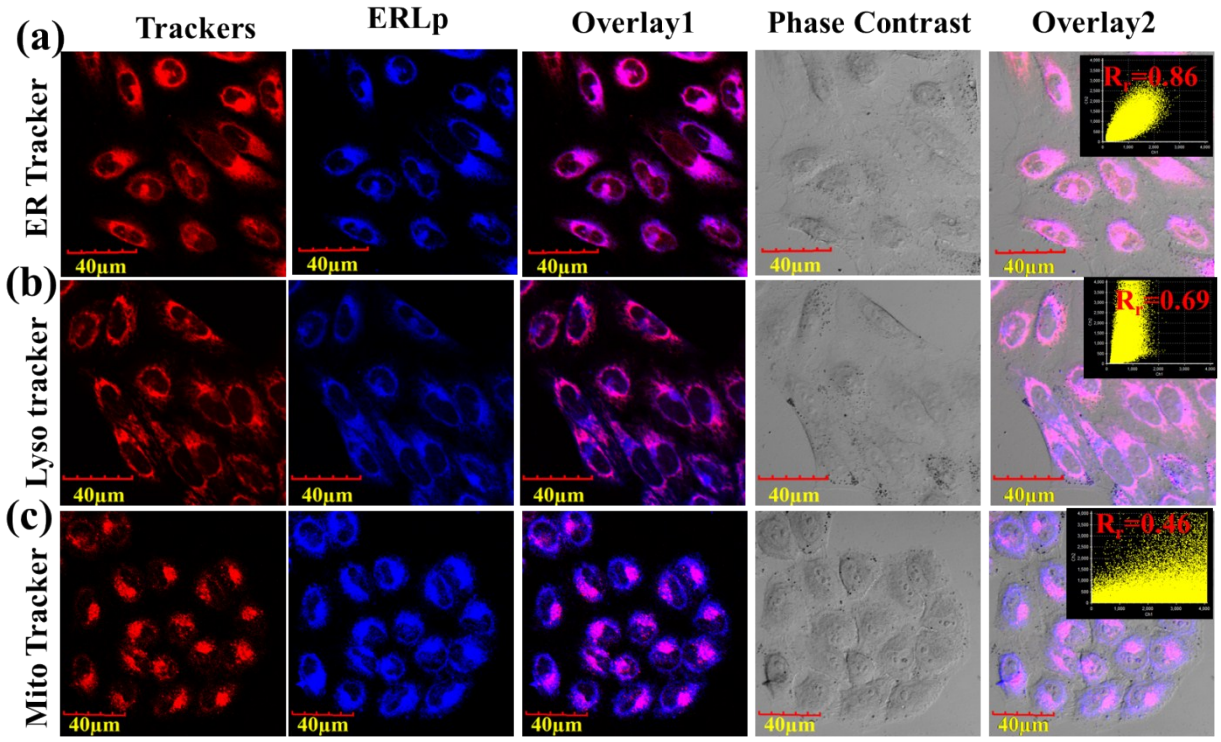

Fig. S22. Co-localization experiment of probe **ERLp** (80 μM, 10 min) with different organelles specific trackers in live Hela cells. (a) ER tracker Red (1μM, 30 min) label endoplasmic reticulum. (b) LysoTracker Red DND-99 (80 nM, 30 min) label lysosomes. (c) MitoTracker Red CMXRos (90 nM for 15 min) label mitochondria. The images from left to right depict organelle trackers (column 1), **ERLp** (column 2), Overlay 1: overlay of the 1st and 2nd columns. Phase contrast (column 4) and Overlay 2: overlay of 1st, 2nd and 4th columns. **ERLp** λ_{ex} = 405 nm; λ_{em} = 415–470 nm; ER tracker Red λ_{ex} = 559 nm; λ_{em} = 570–700 nm.

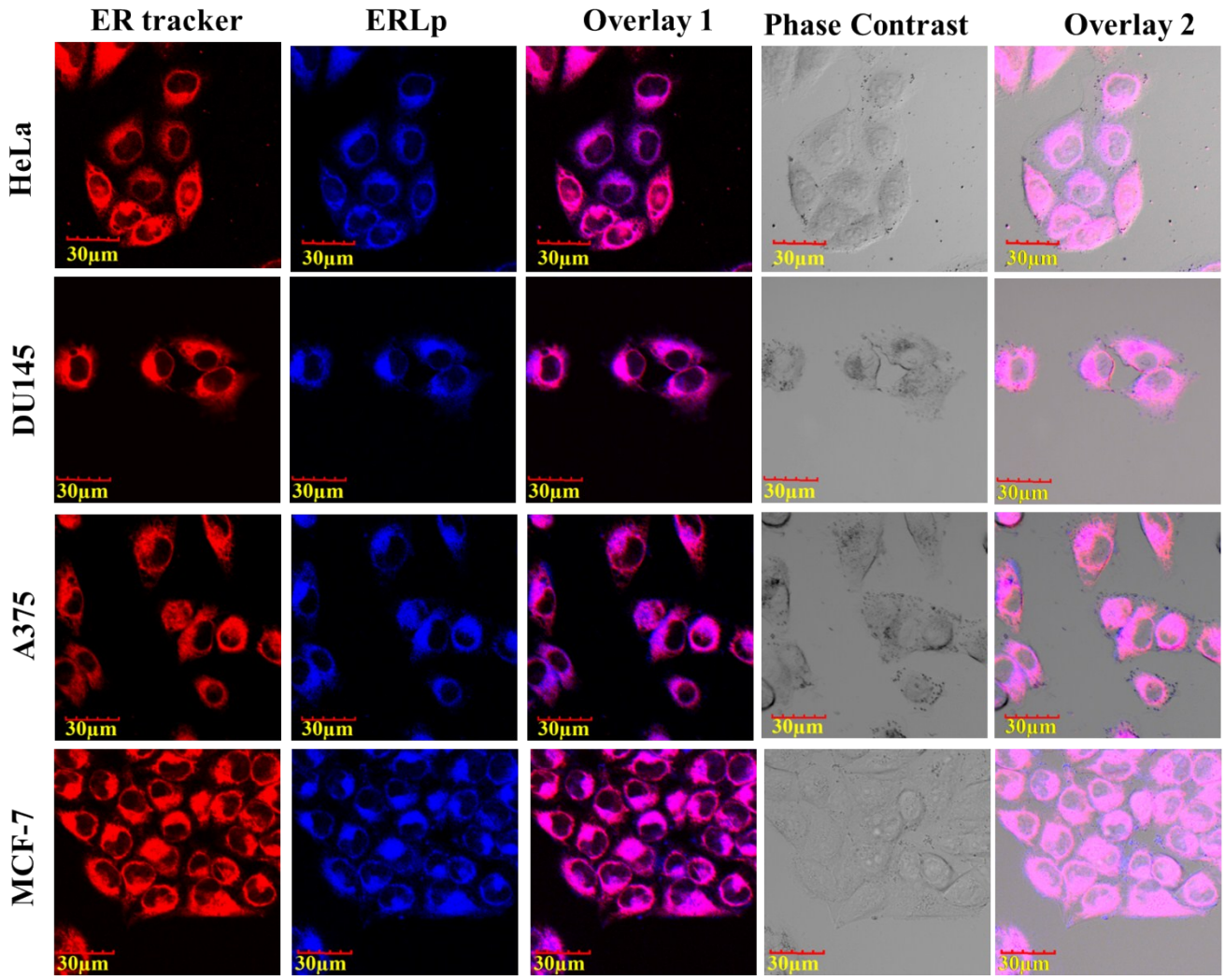

Fig. 23. Endoplasmic Reticulum selective staining of **ERLp** in HeLa, DU145, A375 and MCF-7 cells. All cells were co-treated with **ERLp** (80 μM, 10 min) and ER tracker red (1μM, 30 min). **ERLp**: λ_{ex} = 405 nm; λ_{em} = 415-470 nm; ER tracker red λ_{ex} = 559 nm; λ_{em} = 580-700 nm. Overlay 1: overlay of the 1st and 2nd column. Overlay 2: overlay of the 1st, 2nd and 4th columns. Scale bar: 30 μm.

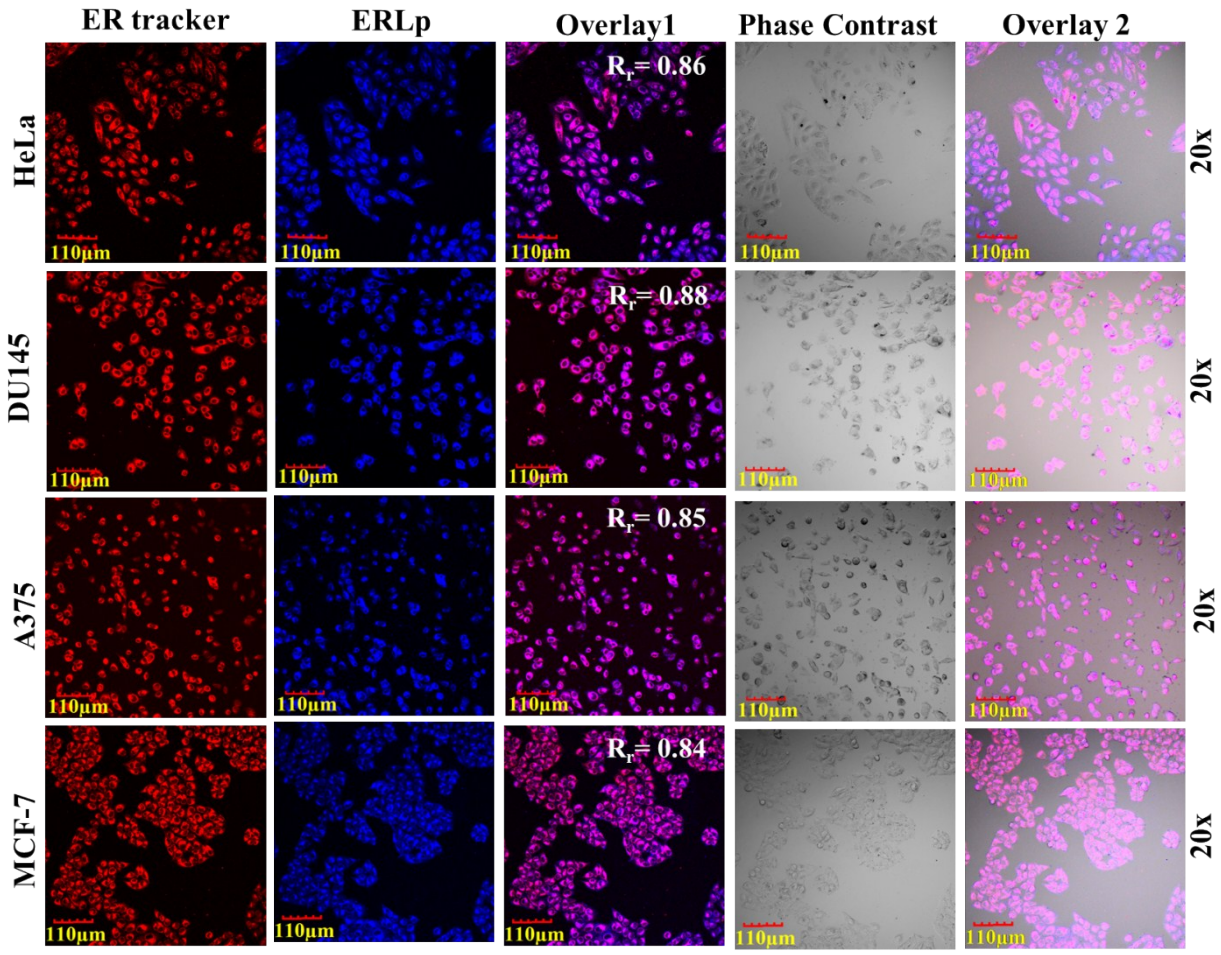

Fig. S24. Endoplasmic reticulum selective live cell imaging of **ERLp** in HeLa, DU145, A375 and MCF-7 cells. All cells were co-stained with **ERLp** (80 µM, 10 min) and ER-tracker red (1µM, 30 min). Pearson colocalization of **ERLp** with ER-tracker red on all four cell lines. **ERLp**: λ_{ex} = 405 nm; λ_{em} = 415–470 nm; ER tracker red λ_{ex} = 559 nm; λ_{em} = 580-700 nm. Overlay 1: overlay of the 1st and 2nd. Overlay 2: overlay of the 1st, 2nd and 4th columns. Scale bar: 110 µm.

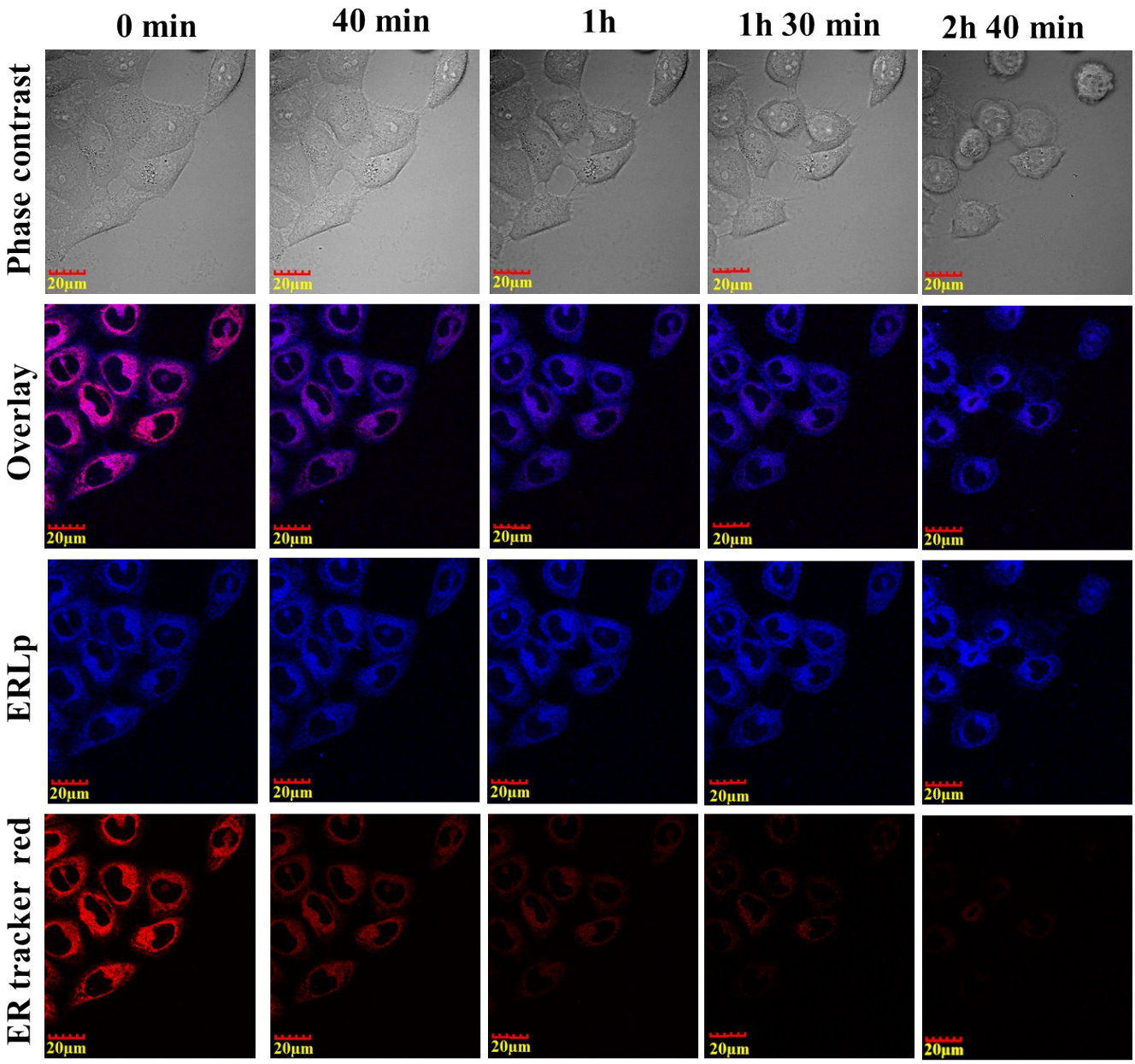

Fig. S25. Endoplasmic Reticulum stress leads to cell apoptosis. Here HeLa cells labeled with **ERLp** (80 μM, 10 min) and co-stained with ER tracker red (1μM, 30 min), then tunicamycin (40 μg/ml) added and the image captured after a different time interval.

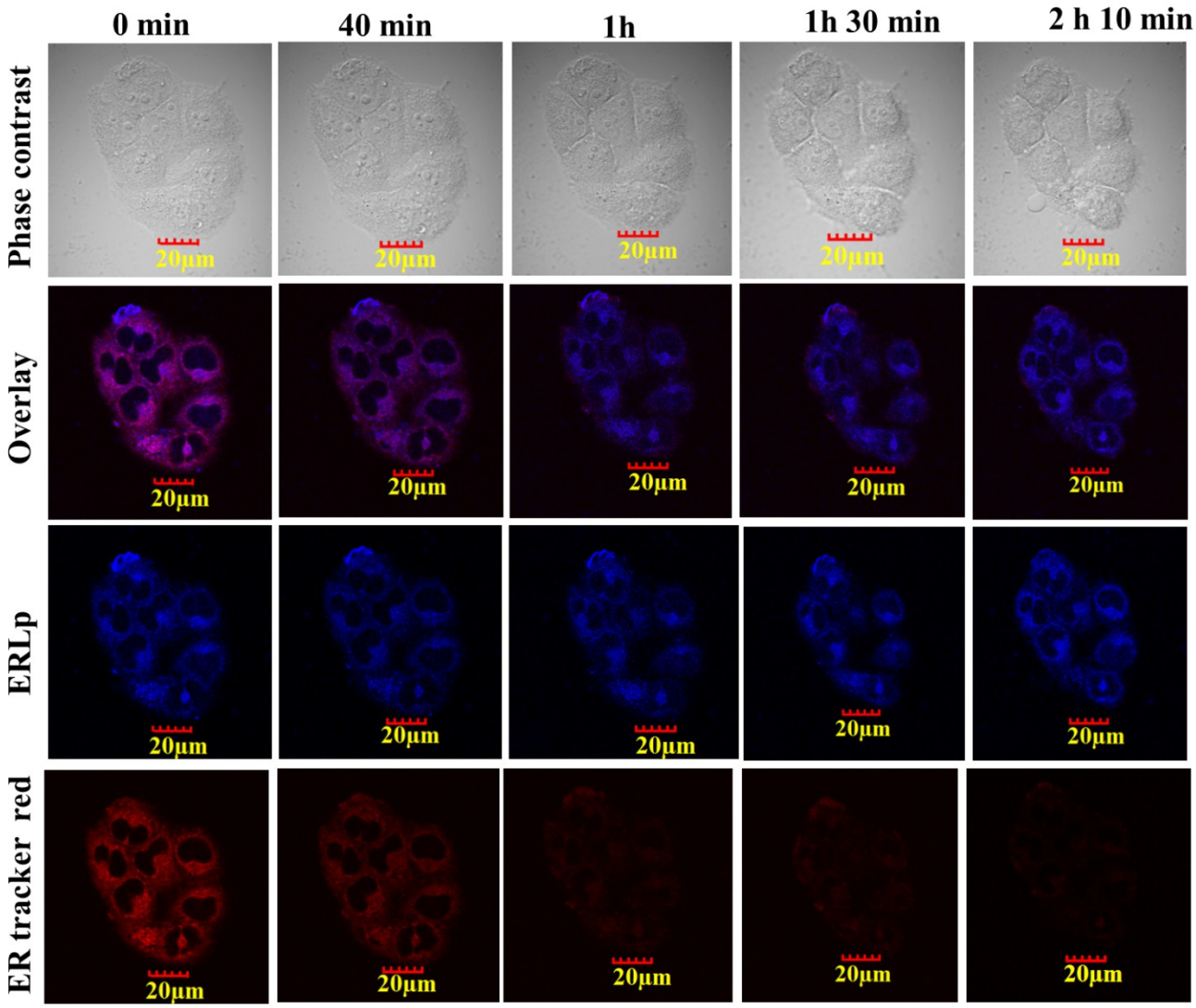

Fig. S26. Endoplasmic Reticulum stress leads to cell apoptosis. Here HeLa cells labeled with **ERLp** (40 µM, 10 min) and co-stained with ER tracker red (1µM, 30 min), then 200 µM Dithiothreitol (DTT) added in media and the image captured after a different time interval.

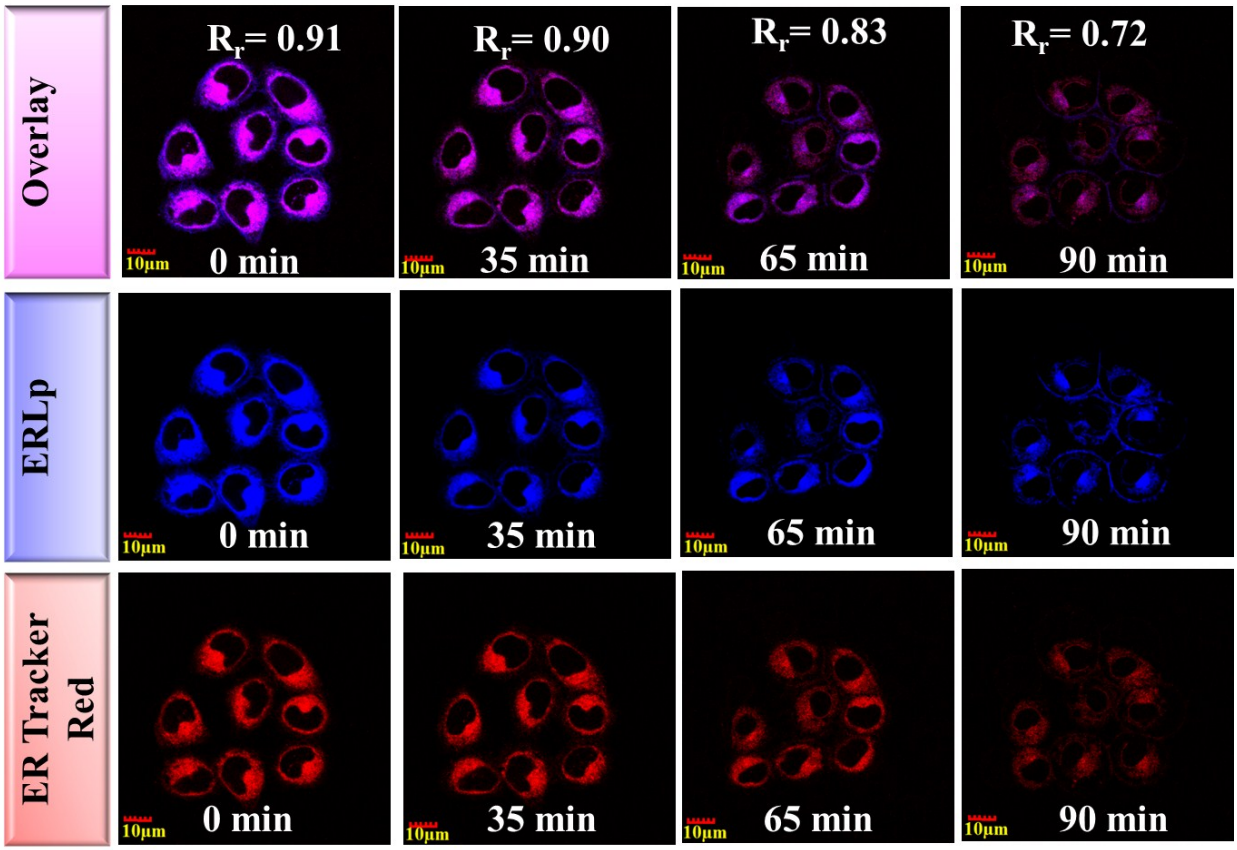

Fig. S27. Time-dependent TPM images of live HeLa cells stained with **ERLp**. HeLa cells stained with **ERLp** and co-labeled with ER tracker red and Pearson's colocalization coefficient measured of each overlay image.

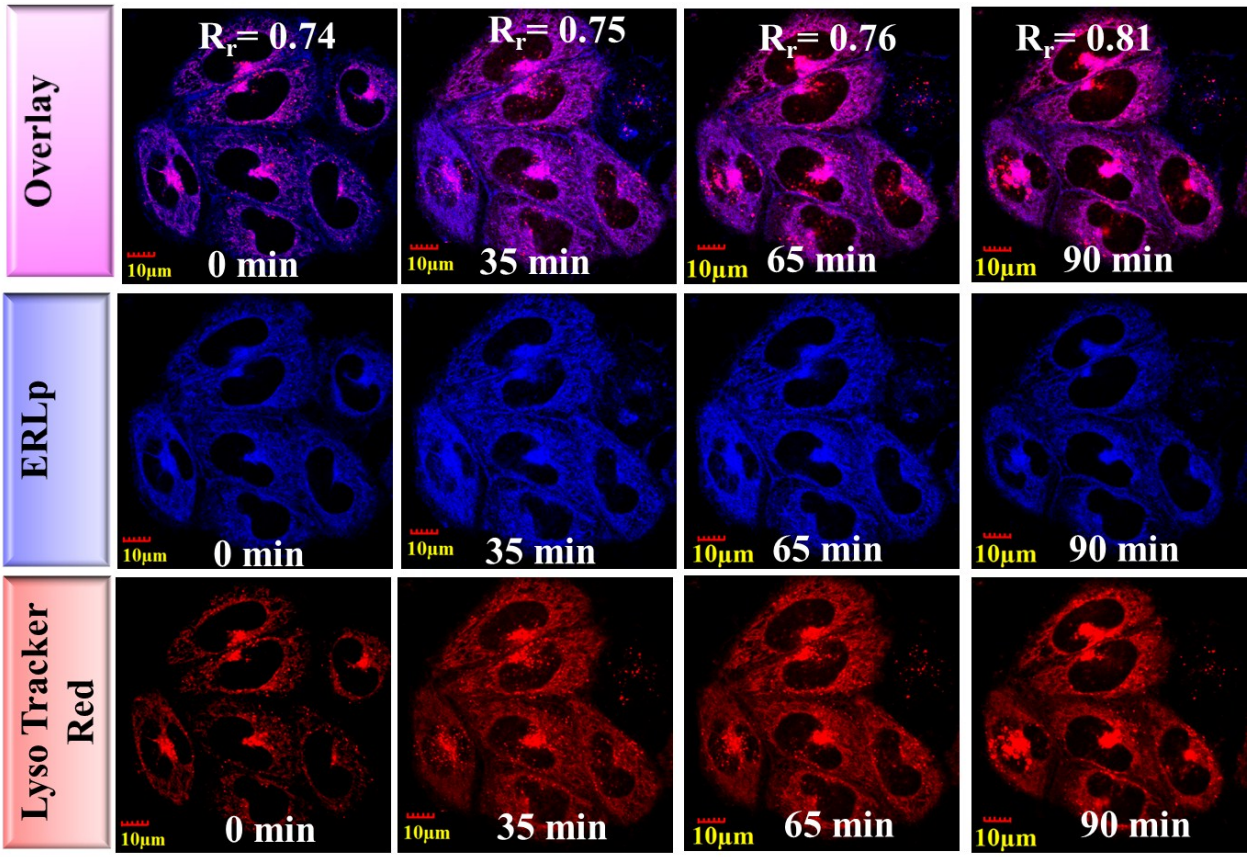

Fig. S28. (a) Time-dependent TPM images of live HeLa cells stained with **ERLp**. HeLa cells stained with **ERLp** and co-labeled with Lyso tracker red DND-99 and Pearson's colocalization coefficient measured of each overlay image.

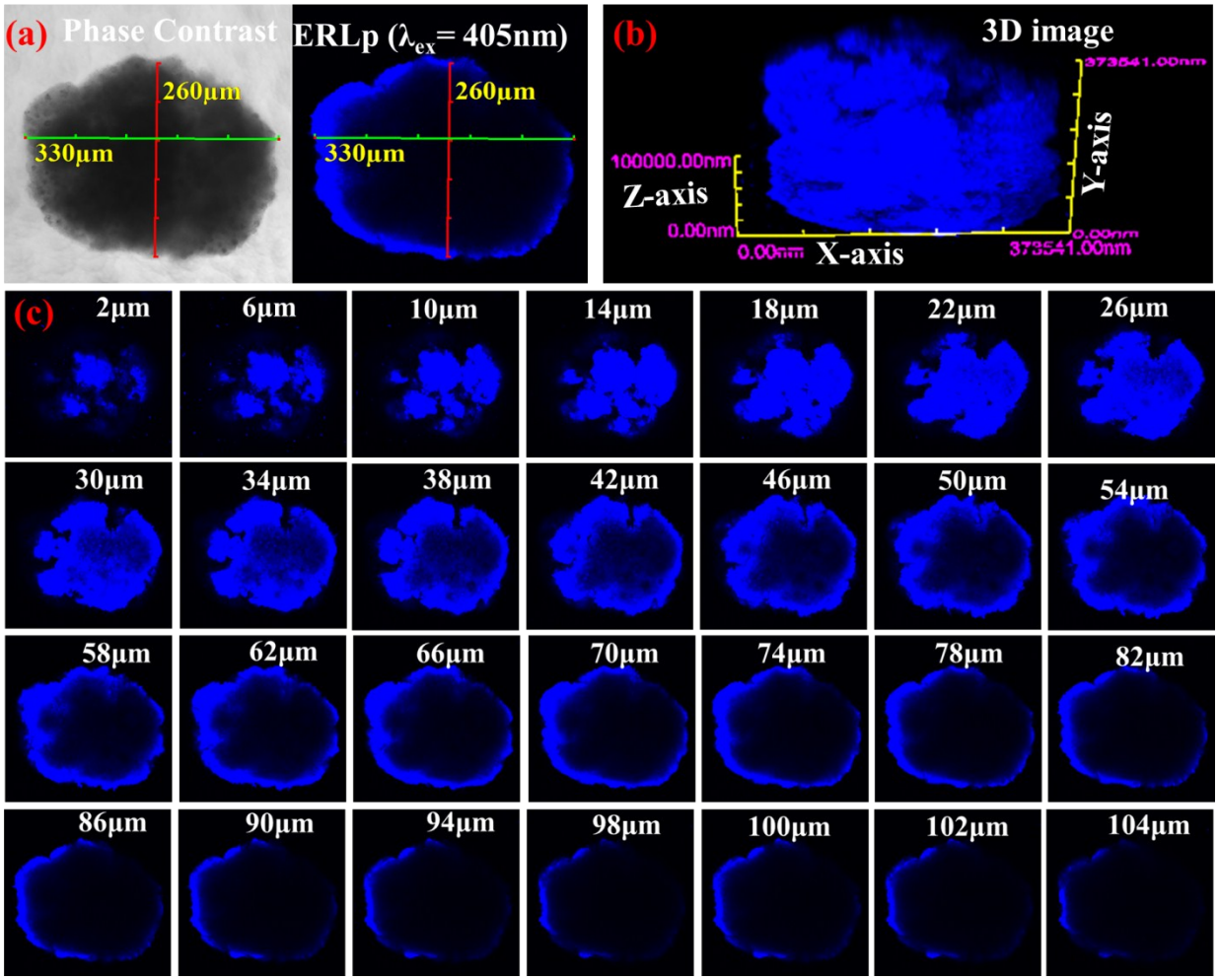

Fig. S29. (a) One-photon fluorescence images of 1st 3D intact tumor spheroid after **ERLp** (80 µM, for 1 h) treatment. (b) The Z-stack 3D images of the intact spheroid. (c) The two-photon Z-stack images were taken after every 2 µm section from the top to bottom spheroid. The images were captured under a 20×1.7z objective λ_{ex} = 405 nm; λ_{em} = 415–470 nm.

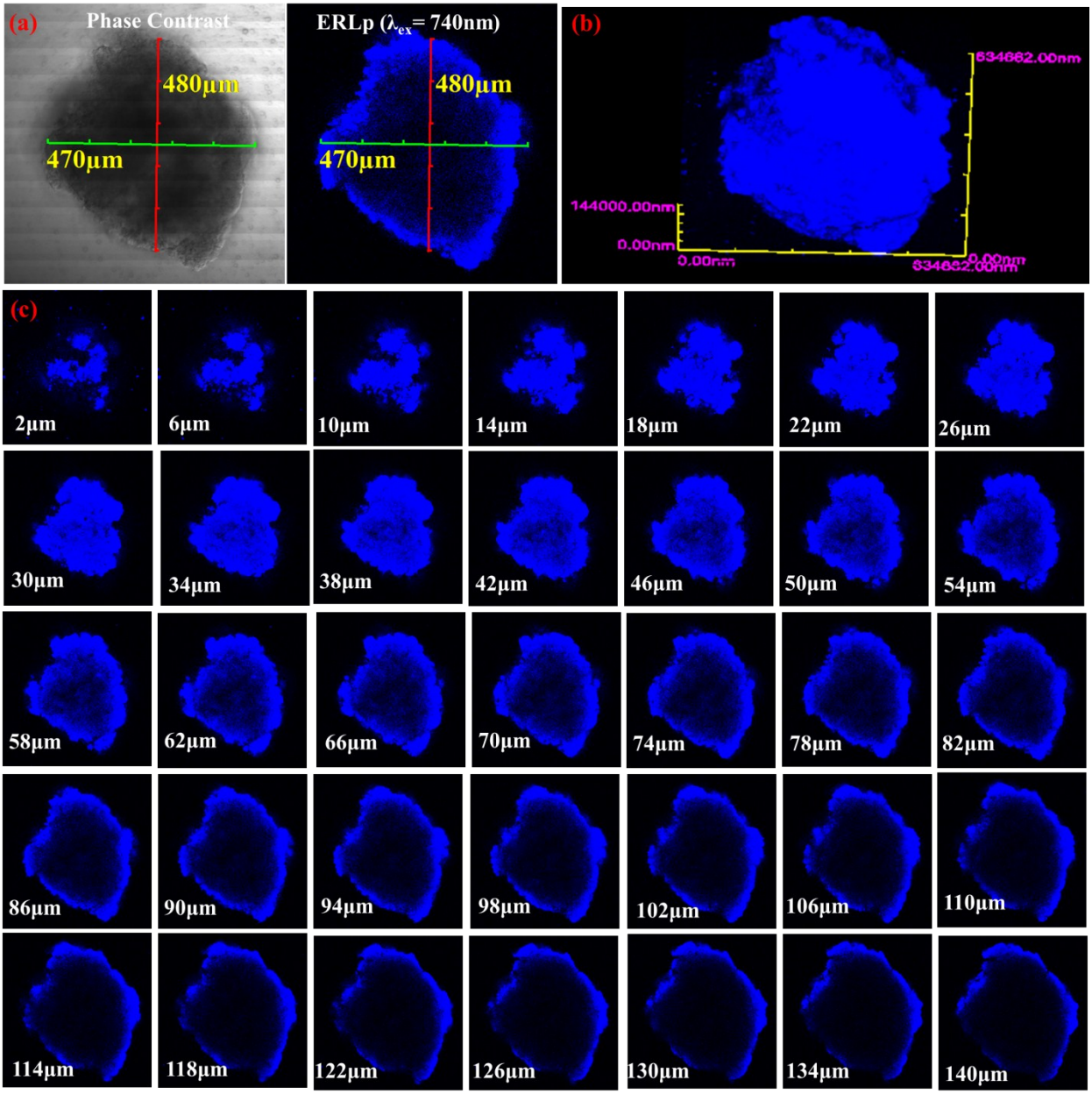

Fig. S30. (a) Two-photon fluorescence images of 2nd 3D intact tumor spheroid after **ERLp** (80 µM, for 1 h) treatment. (b) The Z-stack 3D images of the intact spheroid. (c) The two-photon Z-stack images were taken after every 2 µm section from the top to bottom spheroid. The images were captured under a 20× objective λ_{ex} = 740 nm; λ_{em} = 415–470 nm.

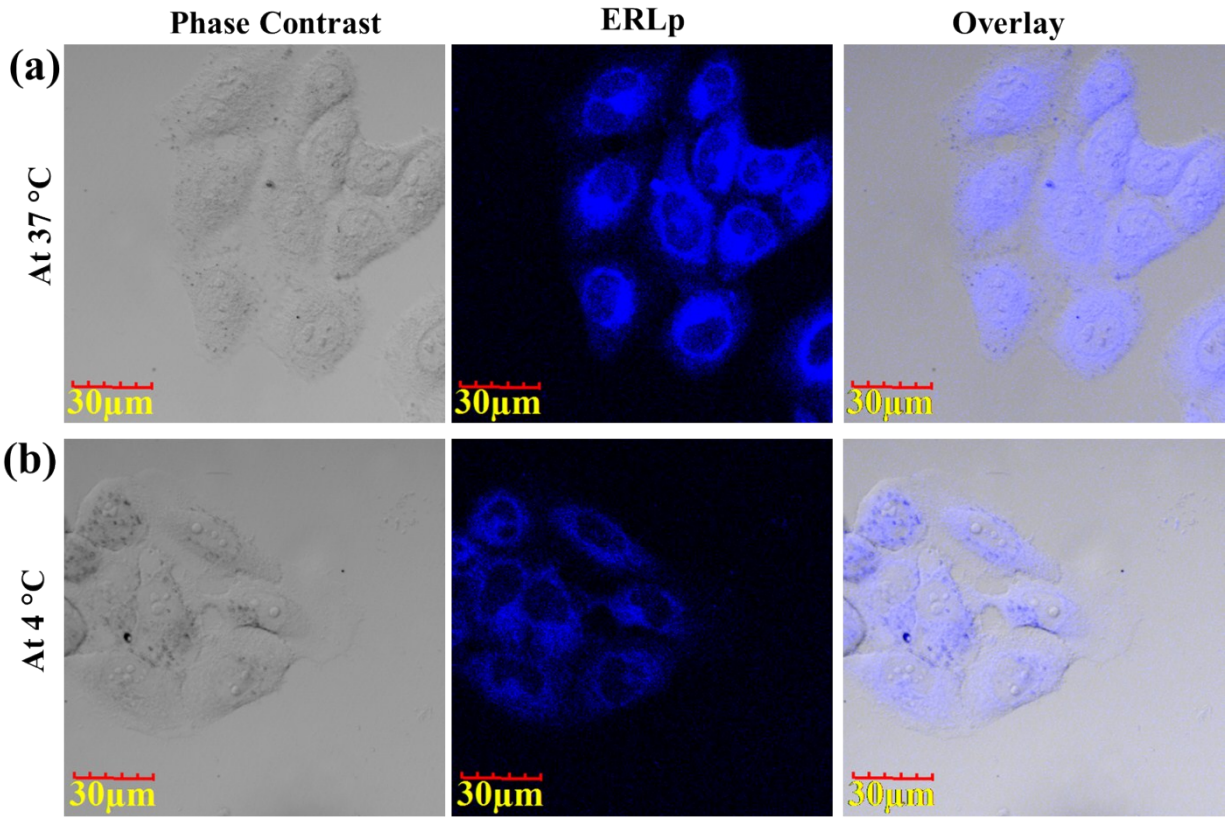

Fig. S31. Live cells imaging under different conditions (a) The HeLa cells were incubated with **ERLp** (80 μM) at 37°C for 50 min. (b) The cells were incubated with **ERLp** (80 μM) at 4°C for 50 min.

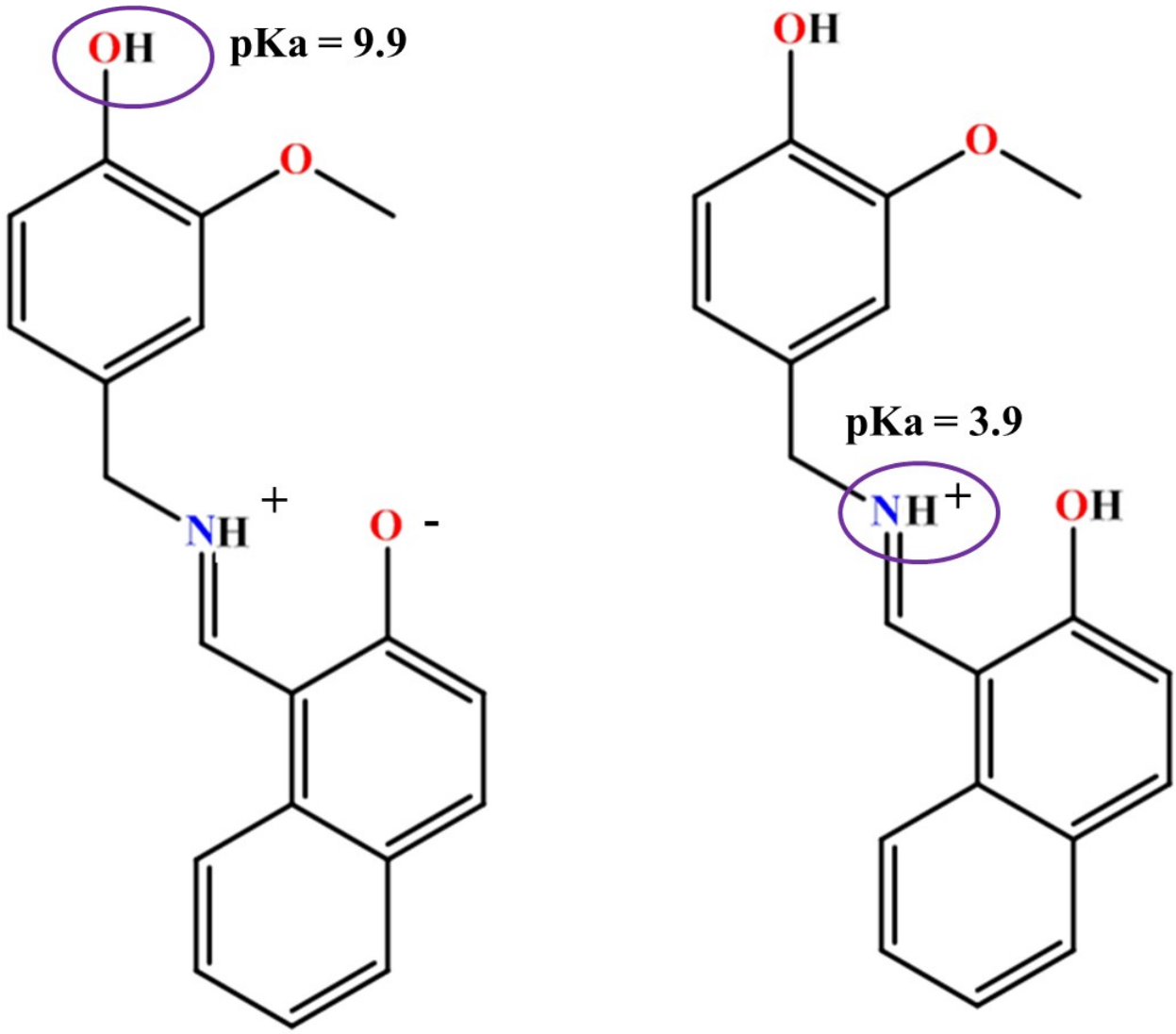

Fig. S32. The ionization state of imine and remote phenolic hydroxyl group of **ERLp.**

Supporting tables

Table S1. Crystallographic data and structural refinements of **ERLp**

Identification code	**ERLp**
Empirical formula	$C_{19}H_{17}NO_3$
Formula weight	307.33
Temperature/K	298
Crystal system	monoclinic
Space group	$P2_1/n$
a/Å	8.31350(10)
b/Å	7.85760(10)
c/Å	24.1360(3)
α/°	90
β/°	99.4900(10)
γ/°	90
Volume/Å^3	1555.09(3)
Z	4
ρ_{calc}g/cm3	1.313
μ/mm^{-1}	0.721
F(000)	648.0
Crystal size/mm^3	0.7 × 0.4 × 0.3
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	7.428 to 142.728
Index ranges	-6 ≤ h ≤ 10, -9 ≤ k ≤ 9, -28 ≤ l ≤ 29
Reflections collected	10584
Independent reflections	2988 [R_{int} = 0.0214, R_{sigma} = 0.0151]
Data/restraints/parameters	2988/0/214
Goodness-of-fit on F^2	1.036
Final R indexes [I>=2σ (I)]	R_1 = 0.0450, wR_2 = 0.1197
Final R indexes [all data]	R_1 = 0.0463, wR_2 = 0.1215
Largest diff. peak/hole / e Å^{-3}	0.17/-0.30
CCDC	1854514

Table S2. Selected bond lengths (Å) and bond angles (°) of **ERLp**

bond lengths (Å)		bond angles (°)	
C1–C2	1.4047 (16)	C4– C1 –C2	119.15(11)
C1–C4	1.3799 (17)	O2– C1– C2	116.43(10)
C1–O2	1.3613 (14)	O2– C1– C4	124.38(11)
C2–C7	1.3776 (16)	C7– C2– C1	119.83(10)
C2–O1	1.3670 (13)	O1– C2– C1	115.01(10)
C3–O1	1.4189 (16)	O1–C2–C7	125.14(10)
C4–C5	1.3942 (18)	C1–C4–C5	120.76(11)
C5–C6	1.3785 (17)	C6–C5–C4	120.11(11)
C6–C7	1.3969 (15)	C5–C6–C7	119.34(11)
C6–C8	1.5070 (15)	C5–C6–C8	124.80(10)
C8– N1	1.4549 (14)	C7–C6–C8	115.81(10)
C9–C10	1.4044 (16)	C2–C7–C6	120.79(11)
C9–N1	1.3081 (14)	N1–C8–C6	115.77(10)
C10–C11	1.4274 (16)	N1–C9–C10	126.15(10)
C10–C15	1.4590 (15)	C9–C10–C11	119.61(10)
C11–C12	1.4371 (18)	C9–C10–C15	119.76(10)
C11–O3	1.2801 (14)	C11–C10–C15	120.59(10)
C12–C13	1.349(2)	C10–C11–C12	117.73(11)
		O3–C11–C10	121.69(11)
		O3–C11–C12	120.58(11)
		C13–C12–C11	121.43(12)
		C9–N1–C8	122.32(10)
		C2–O1–C3	117.08(10)

Table S3. The quantum yield of **ERLp** and ER tracker red

Sensor	**Solvent**	**Excitation**	**Emission**	**Quantum yield(ΦF)**
Trptophan (standard)	H_2O	280 nm	300-380 nm	0.14
ERLp	H_2O	280 nm	360-494 nm	0.12
Quinine Sulfate (standard)	0.1 M H_2SO_4	350 nm	400-600 nm	0.54
ER traker red	H_2O	587 nm	598-650 nm	0.35

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Movies

Movie S1. Photostability of **ERLp** on HeLa cells.

Movie S2. Photostability of ER tracker red on HeLa cells.

Movie S3. Two-photon fluorescence imaging of 1st 3D tumor spheroid 1 after treatment with **ERLp** (80 μM for 1 h). The two-photon Z-stack images were captured after every 2 μm section from the top to bottom of tumor spheroid.

Movie S4. Ono-photon fluorescence imaging of 1st 3D intact tumor spheroid after treatment with **ERLp.** The One-photon Z-stack images were captured after every 2 μm section from the top to bottom of tumor spheroid 1.

Movie S5. Two-photon fluorescence imaging of 2nd 3D intact tumor spheroid (big size) after treatment with **ERLp**. The two-photon Z-stack images were captured after every 2 μm section from the top to bottom of tumor spheroid 2.