Electronic Supplementary Information (ESI)

Lysosomal tracking with a cationic naphthalimide using multiphoton fluorescence lifetime imaging microscopy

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1. General Methods

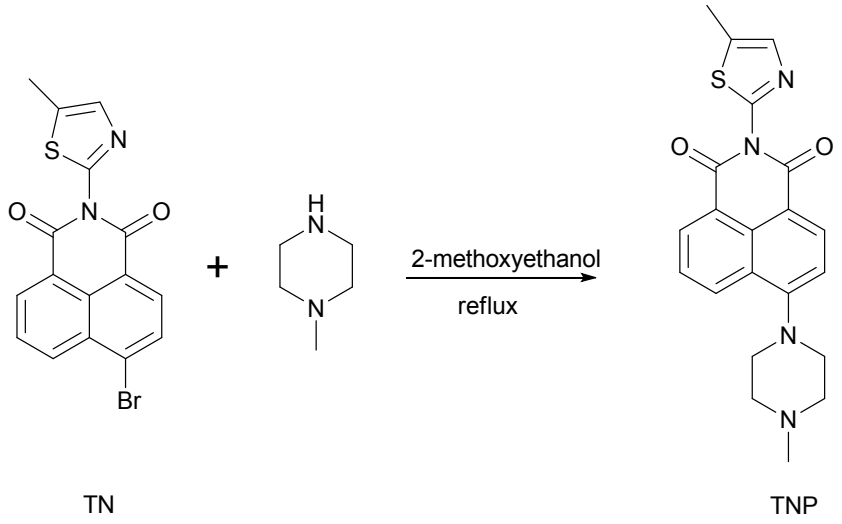
All chemical reagents and solvents were analytical grade and purchased from commercial suppliers.

6-bromo-2-(5-methylthiazol-2-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione,6-(4-methylpiperazin-1-yl) -2-(5-methylthiazol-2-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione and6-(bis(pyridin-2-ylmethyl)amino)-2-(5-methylthiazol-2-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione were prepared by the established literature procedure.[1] ^{1}H NMR and ^{13}C NMR spectra were recorded on the Bruker AV-300 spectrometer with chemical shifts reported in ppm (in $CDCl_3$, TMS as internal standard) at room temperature. Mass spectra were measured on a HP 1100 LC-MS spectrometer.

UV-vis absorption spectra were recorded on a Perkin Elmer spectrophotometer. Fluorescence measurements were performed on a Perkin Elmer Luminescence spectrophotometer LS 50B, utilising sterna silica (quartz) cuvettes with 10 mm path length and four sides polished. Spectral-grade solvents were used for measurements of UV-vis absorption and fluorescence.

6-bromo-2-(5-methylthiazol-2-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (TN)

Anhydride naphthalene (4.00 g, 14.44 mmol) was dissolved in absolute acetate acid (30 mL). An excess of 2-thizolamine (2.00 g, 17.52 mmol) was added, after having refluxed for 4 h, the mixture was cooled and the precipitated solids were filtered and recrystallized from toluene to give yellow solid (3.23 g, 60% yield).^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ_H 8.61 (d, J = 1.2 Hz, 1H), 8.59 (d, J = 1.2 Hz, 1H), 8.41 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 7.8 Hz, 1H), 7.83 (m, 1H), 7.50 (d, J = 1.2 Hz, 1H), 2.51 (d, J = 1.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): δ_C 163.4, 163.3, 152.8, 138.9, 137.6, 134.3, 132.8, 131.9, 131.4, 131.4, 130.9, 129.4, 128.3, 122.6, 121.7. HRMS (ESI μTOF) m/z calcd for $C_{16}H_9N_2O_2SBr$ $[M + H]^+$ 372.9646, found 372.9678.



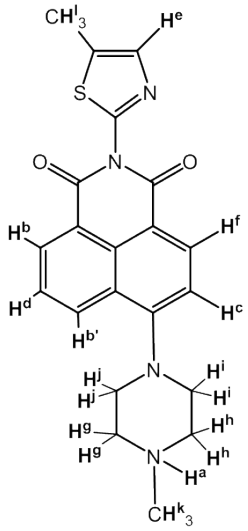
TN TNP

Scheme S1. Schematic representation of the synthesis of **TNP**.

6-(4-methylpiperazin-1-yl)-2-(5-methylthiazol-2-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione(TNP)

N-thizolamine-4-bromine-1, 8-naphthalimide (0.60 mg, 1.61 mmol) and 1-methylpiperazine (0.24 g, 2.41 mmol) were added into 30 mL 2-methoxyethanol. After added 3 mL triethylamine, the mixture was refluxed for 48 h. After completion of the reaction, the solvent was removed *in vacuo* and the residue was dissolved in DCM and washed with water (3×10 mL). The organic phase was dried with sodium sulfate. The residue was purified with column chromatography (silica gel, DCM–MeOH, 50: 1, v/v), and a red solid was obtained (400 mg, 64%). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 8.55 (q, 1H), 8.49 (d, J = 8.1 Hz, 1H), 7.65 (q, 1H), 7.48 (d, J =1.2 Hz, 1H), 7.16 (s, 1H), 3.30 (t, J = 4.2, 4H), 2.73 (s, 4H), 2.50 (d, J = 0.9 Hz, 3H), 2.41 (s, 3H). ^{13}C NMR (75 MHz, DMSO, ppm): δ_C 164.0, 163.4, 156.7, 153.8, 138.6, 137.8, 133.0, 131.8, 131.5, 130.1, 126.4, 125.7, 122.7, 115.5, 115.3, 54.9, 52.9, 46.1, 12.4.HRMS (ESI μTOF) m/z calcd for $C_{21}H_{20}N_4O_2S$ $[M + Na]^+$ 415.1205, found 415.1214.

N-methyl protonated 6-(4-methylpiperazin-1-yl) -2-(5-methylthiazol-2-yl)- 1H-benzo[de]isoquinoline-1,3(2H)-dione [TNPH**]$^+$**



Scheme S2

TNP (6 mg) was dissolved in 7 ml of tetrahydrofuran (THF). After 5 minutes the solid yellow was completely dissolved, and 20 µL (ca. 1.1 equivalent) of HCl (37% concentration) were added to the solution. The solution turned almost immediately into a neon-yellow, and after 10 minutes, and a neon-yellow solid crashed out. The solid was filtered off and washed with THF (2 x 10 mL) and Ethyl ether (3 x 10 mL), and dried under reduced pressure. ^{1}H NMR (500 MHz, 298.5K, DMSO-d_6): δ = 10.44 (1H, H$^{\mathbf{a}}$, bm), 8.56 (2H, H$^{\mathbf{b}}$/H$^{\mathbf{b'}}$, dd, $^2J_{Hb'\text{-}Hd}$ = 10 Hz, $^2J_{Hb\text{-}Hd}$ = 8 Hz), 8.47 (2H, H$^{\mathbf{c}}$, $^2J_{Hc\text{-}Hf}$ = 8 Hz), 7.91 (1H, H$^{\mathbf{d}}$, t,), 7.58 (1H, H$^{\mathbf{e}}$, d, $^4J_{He\text{-}Hl}$ = 1 Hz), 7.50 (1H, H$^{\mathbf{f}}$, d), 3.73 (2H, H$^{\mathbf{g}}$, m), 3.63 (2H, H$^{\mathbf{h}}$, m), 3.50, (2H, H$^{\mathbf{i}}$, m), 3.36 (2H, H$^{\mathbf{j}}$, m mainly overlapping the H_2O signal), 2.94 (3, H$^{\mathbf{k}}$, bs), - 2.54 (3H, H$^{\mathbf{l}}$, bs) ppm. ^{13}C NMR (125 MHz, 298.5K, DMSO-d_6): δ = 164.1, 163.5, 154.9, 153.8, 138.7, 138.0, 132.9, 131.7, 130.1, 127.0, 126.0, 123.0, 116.9, 116.5, 55.4, 53.1, 49.9, 12.5 ppm. MS (ESI µTOF) m/z for $C_{21}H_{21}N_4O_2S$ [M]$^+$ calcd 393.1380, found 394.1453.

2. X-Ray crystallography

Intensity data for k14sip1 was collected at 150 K on a Nonius KappaCCD diffractometer equipped with an Oxford Cryostream, using graphite monochromated MoKα radiation (λ= 0.71073 Å). Data were processed using the Nonius Software.[2]. A symmetry-related (multi-scan) absorption correction had been applied. Crystal parameters and details on data collection, solution and refinement for the complexes are provided in Table 1. Structure solution, followed by full-matrix least squares refinement was performed using the WINGX-1.80 suite of programs throughout.[3] Non-hydrogen atoms were refined with anisotropic displacement parameters. The hydrogen atoms of water and N were located in the difference Fourier map and freely refined, while the other hydrogen atoms were included in calculated positions and refined with isotropic thermal parameters riding on the corresponding parent atoms.

Relative fluorescence quantum yields (QY) determination

UV/visible and fluorescence spectra were recorded on Perkin Elmer Lambda 650 UV/vis and a Perkin Elmer LS55 Luminescence spectrometer, respectively. Room temperature fluorescence QY was calculated according to the following equation[4]:

$$\phi s = \phi r * \frac{Ar}{As} * \frac{Es}{Er} * \frac{Ir}{Is} * \frac{{n_s}^2}{{n_r}^2}$$

In this equation subscripts *r* refers to the reference ([Ru(bPy)$_3$]Cl air saturated solution in water),[5] while *s* is referred to [**TNP**H]$^+$. Φr and Φs are the fluorescence QY [Ru(bPy)$_3$]Cl of (0.028), and unknown [**TNP**H]$^+$. A is the absorbance of the solution, E is the corrected emission intensity, I is the relative

intensity of the exciting light and n is the average refractive index of the solutions.

3. UV-vis spectra of TNP

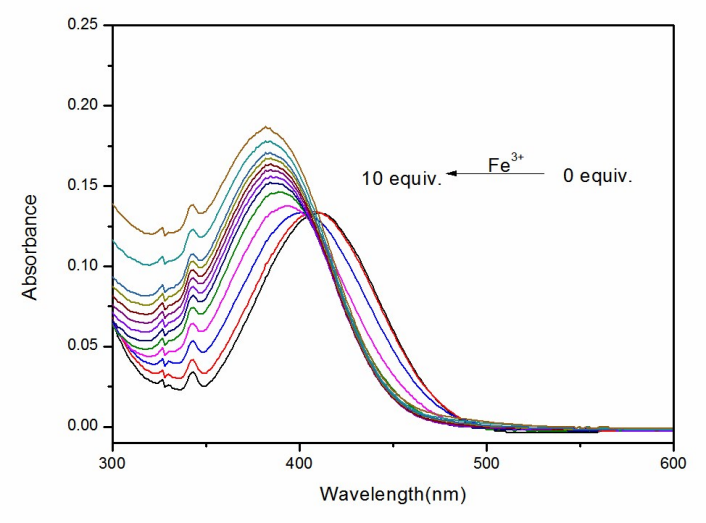


Figure S1. Absorption spectra of **TNP** (10 μM) upon titration with Fe^{3+} from 0 to 10 equiv in ethanol–water (80:20, v/v) with a buffer solution of HEPES (10 mM, pH = 7.4).

4. 2D fluorescence mapping of $[TNPH]^+$

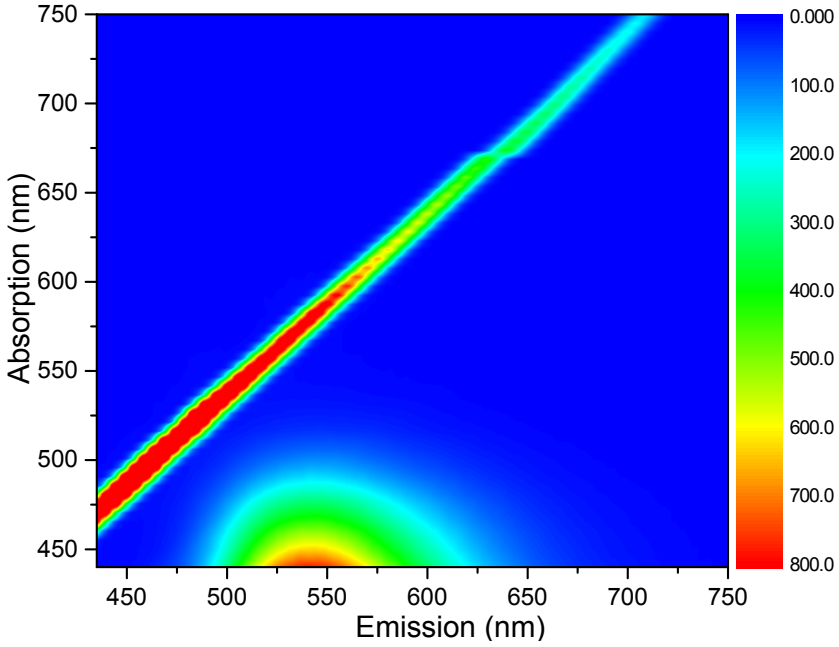


Figure S2 2D fluorescence mapping of [**TNP**H]$^{+}$ obtained from TNP with a buffer solution of HEPES (2×10^{-6} M, pH = 7.4).

5. Job's plot of TNP

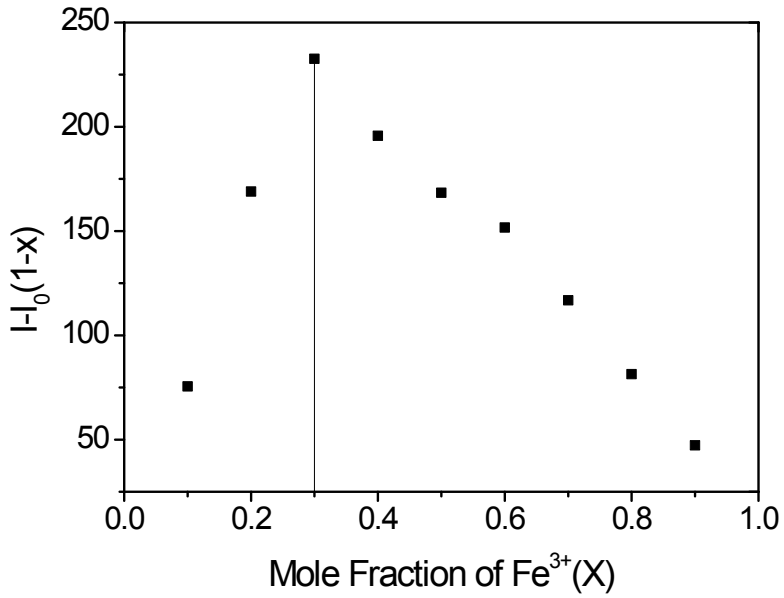
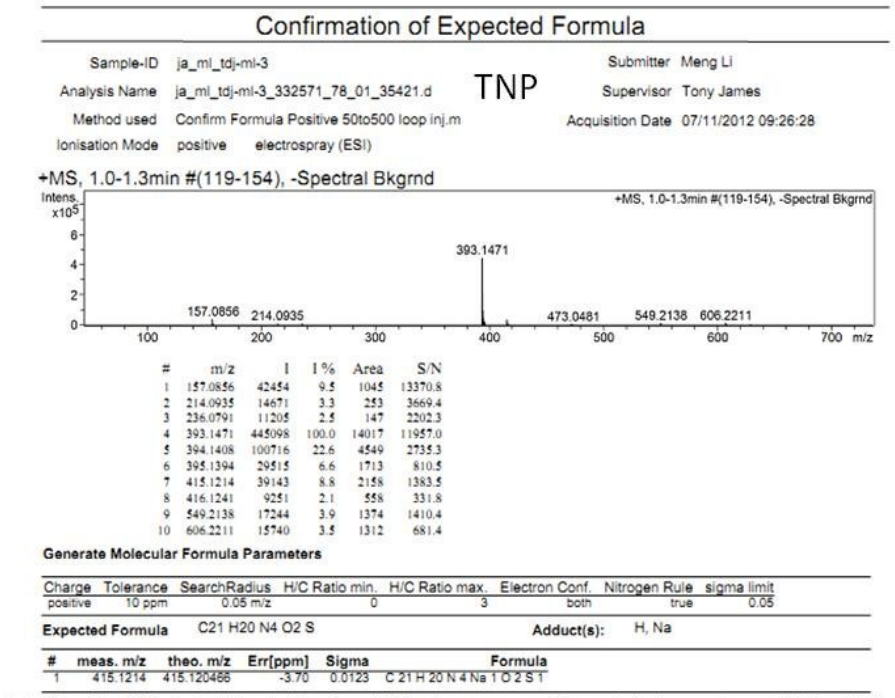
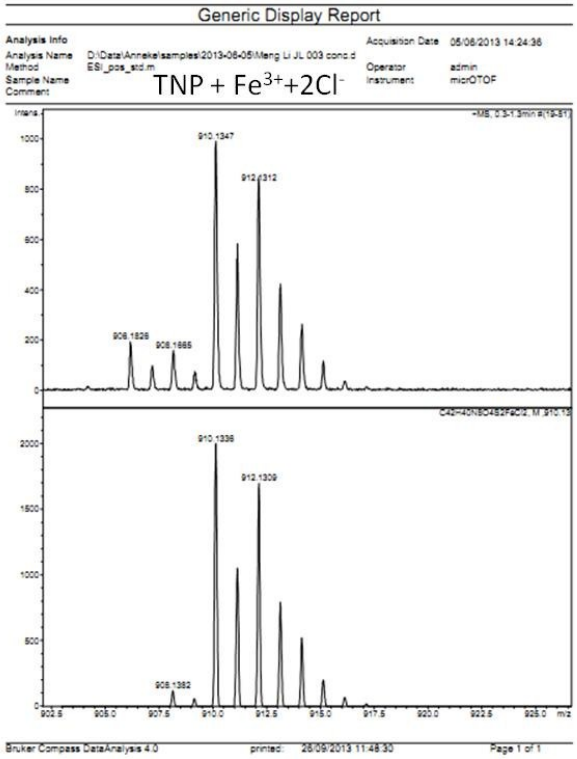


Figure S3 Job's plot of sensor **TNP** in ethanol–water (80:20, v/v) with a buffer solution of HEPES (10 mM, pH = 7.4). The total concentration of sensor **TNP** and Fe^{3+} is 50.0 μM

6. Mass spectra



(a)



(b)

Figure S4. Mass spectra of **TNP** (a) and **TNP + Fe^{3+}** (b) systems

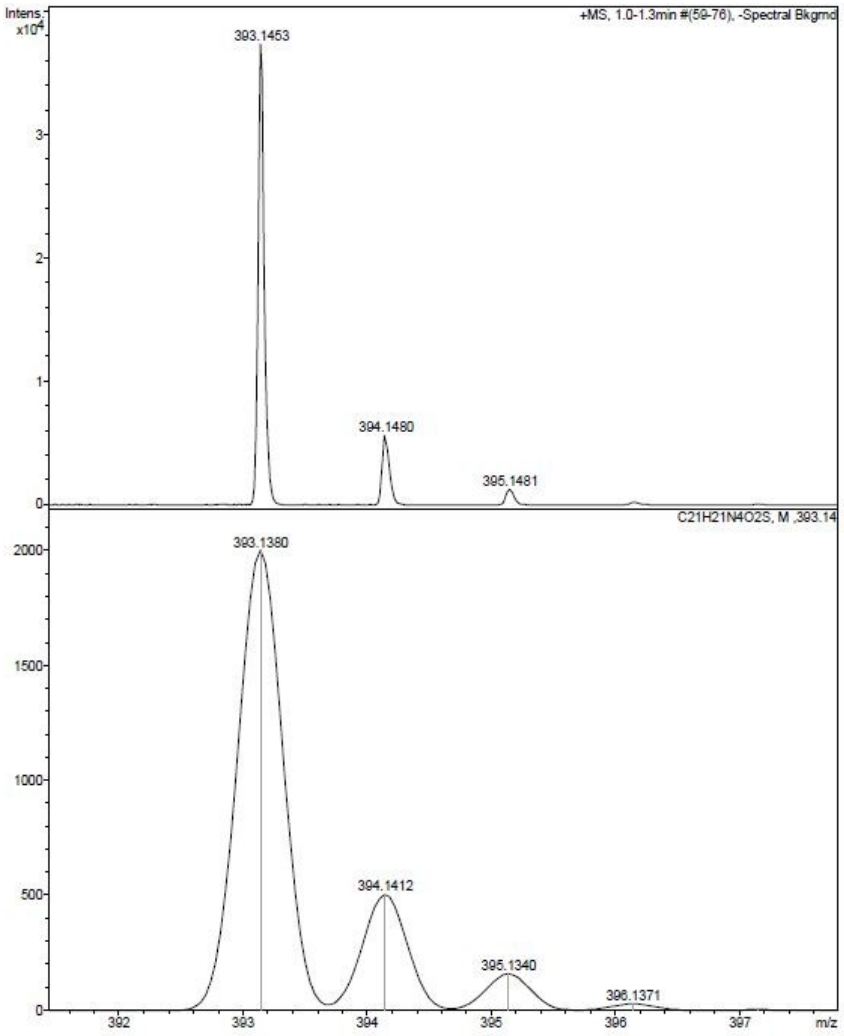


Figure S5. Mass spectra of [**TNP**H]$^+$ and corresponding isotope fitting

7. Association constant

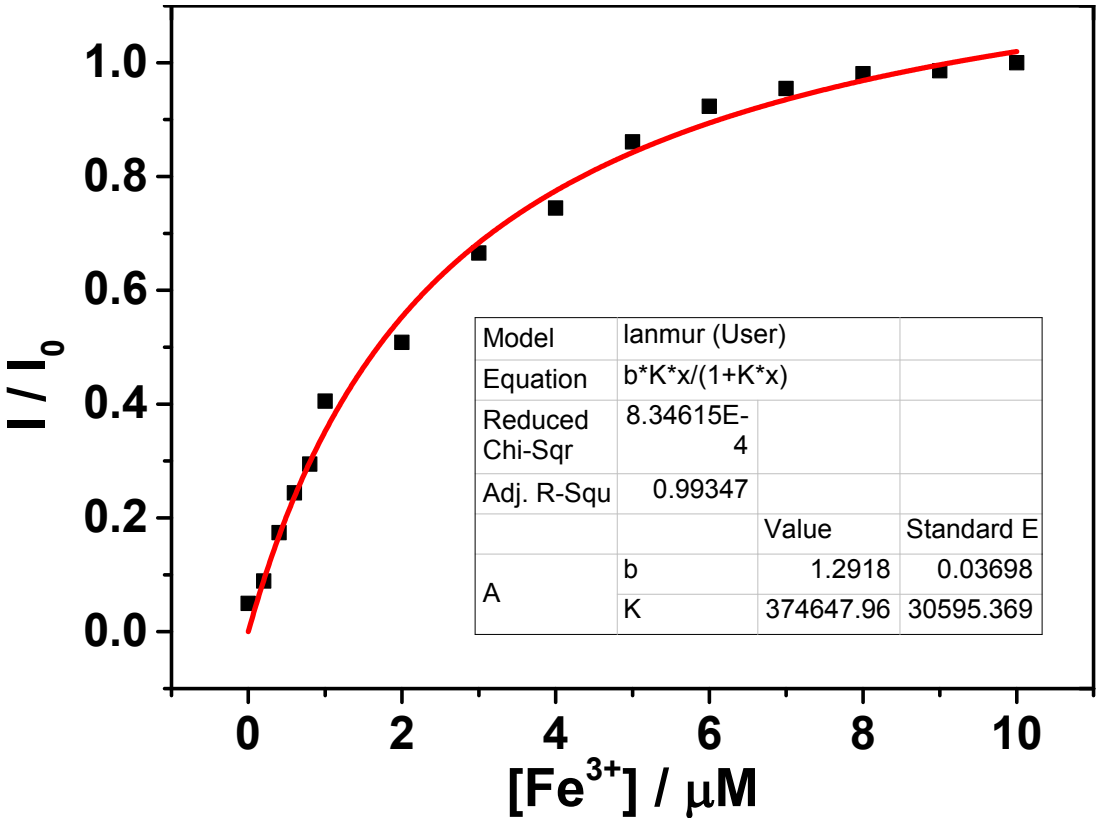


Figure S6. The response of fluorescence signals to Fe^{3+} concentrations, a non-linear regression curve was then fitted to these fluorescent intensity data. Therefore, the association constant was calculated by the formula [y= b*k*x/ (1+k*x)] and gave a result as (3.75± 0.31) × 10^5 M^{-1}.

8. pH dependency of TNP

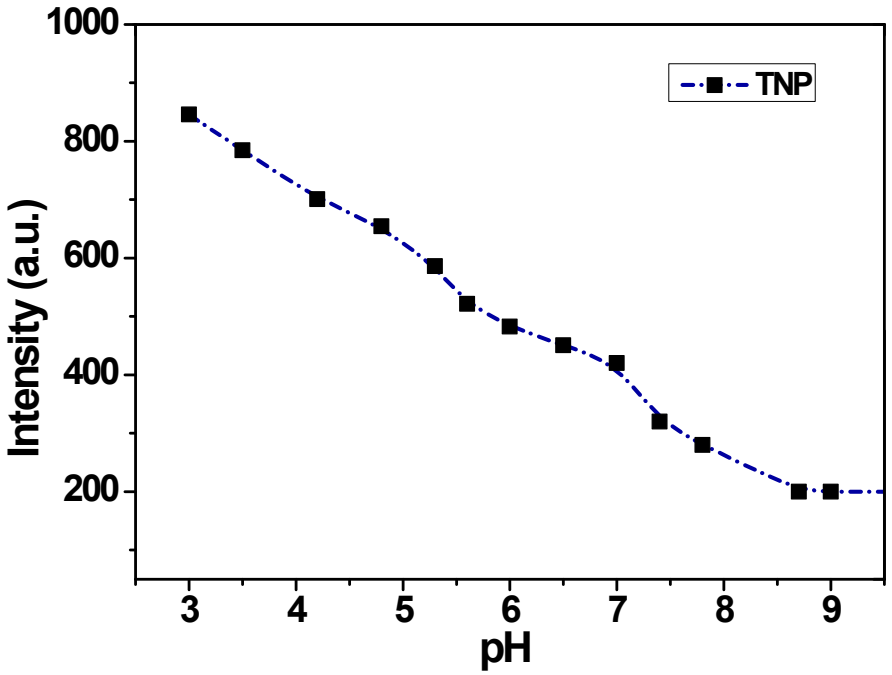


Figure S7. Effect of pH on fluorescence intensity of **TNP** in ethanol–water (80:20, v/v) (λ_{ex} = 403 nm, λ_{em} = 515 nm)

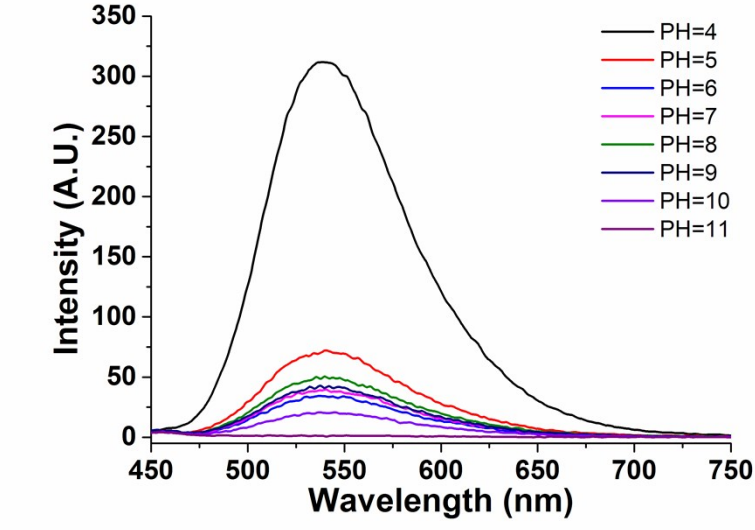


Figure S8. Effect of the pH (4.0 – 11.0) on the fluorescence emission intensity of [**TNP**H$^+$]Cl$^-$ in a H_2O/DMSO mixture (1:99 v/v) (λ_{ex} = 400 nm).

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9. NMR

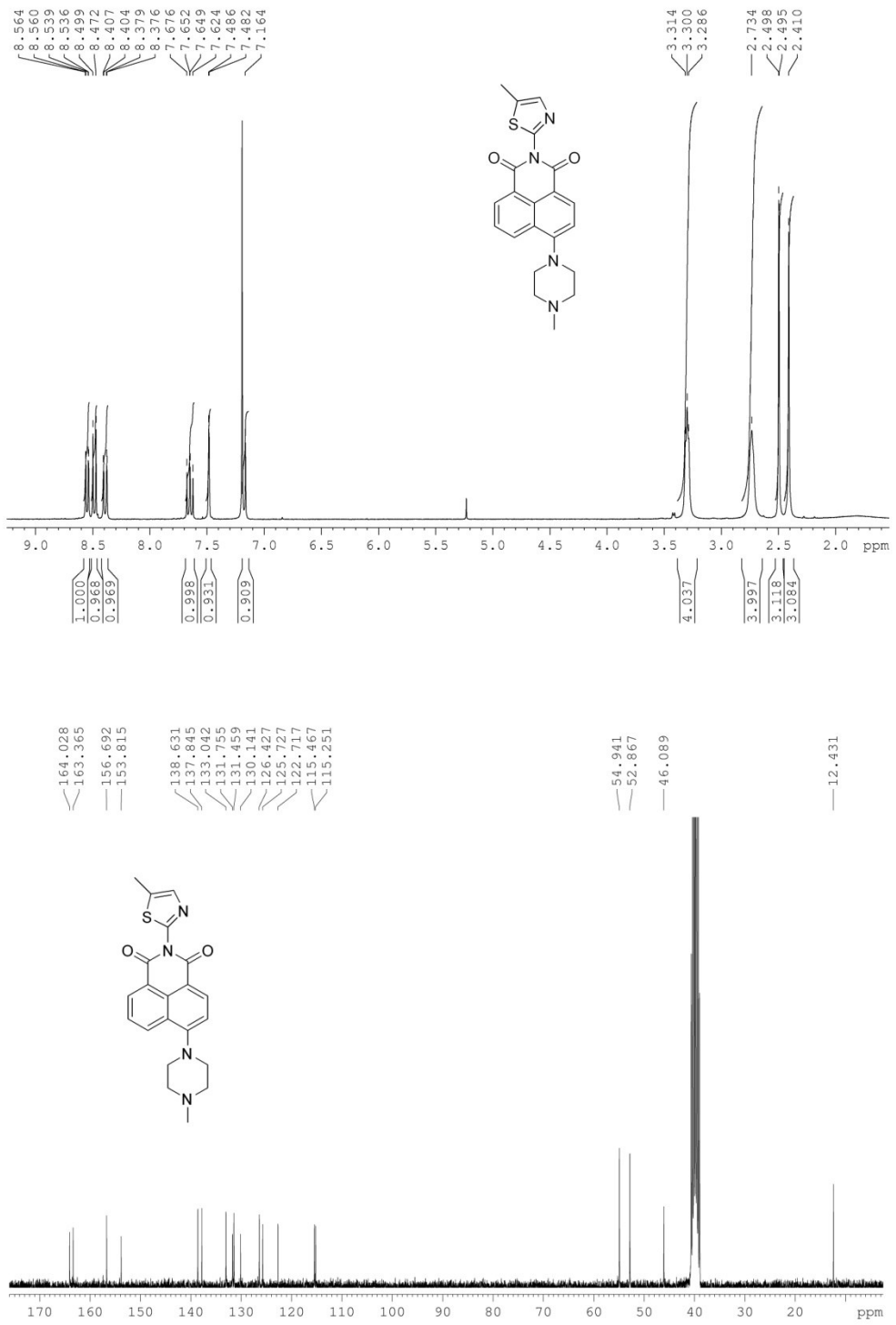

Figure S9. ^{1}H NMR and ^{13}C NMR spectra of **TNP**.

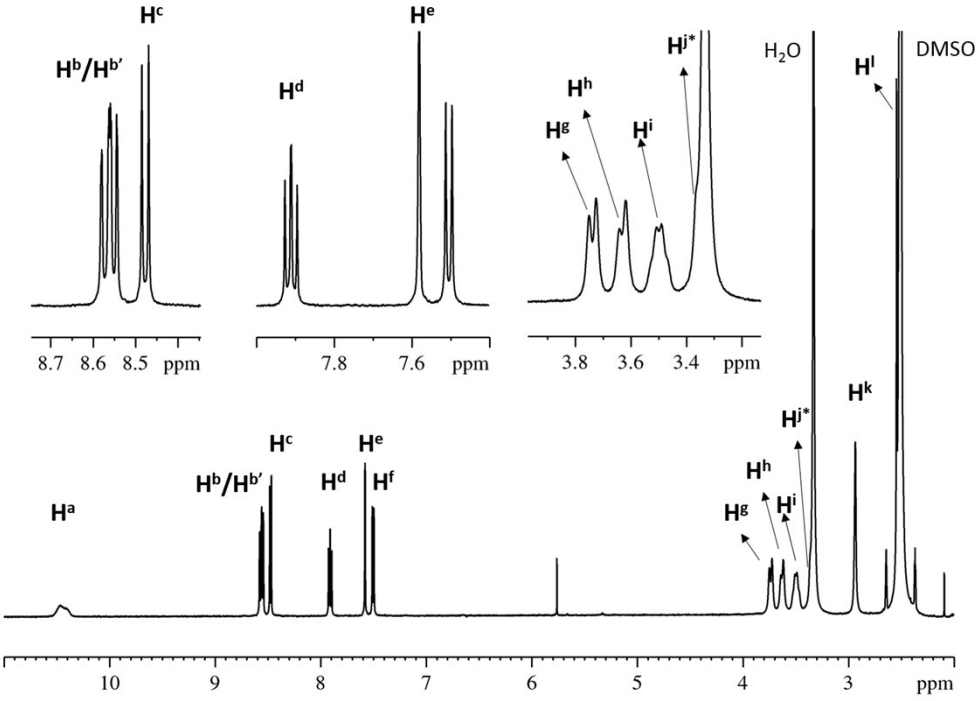

Figure S10. ^{1}H NMR spectrum (500 MHz, 298.5K, DMSO-d_6) of [**TNP**H]$^{+}$.

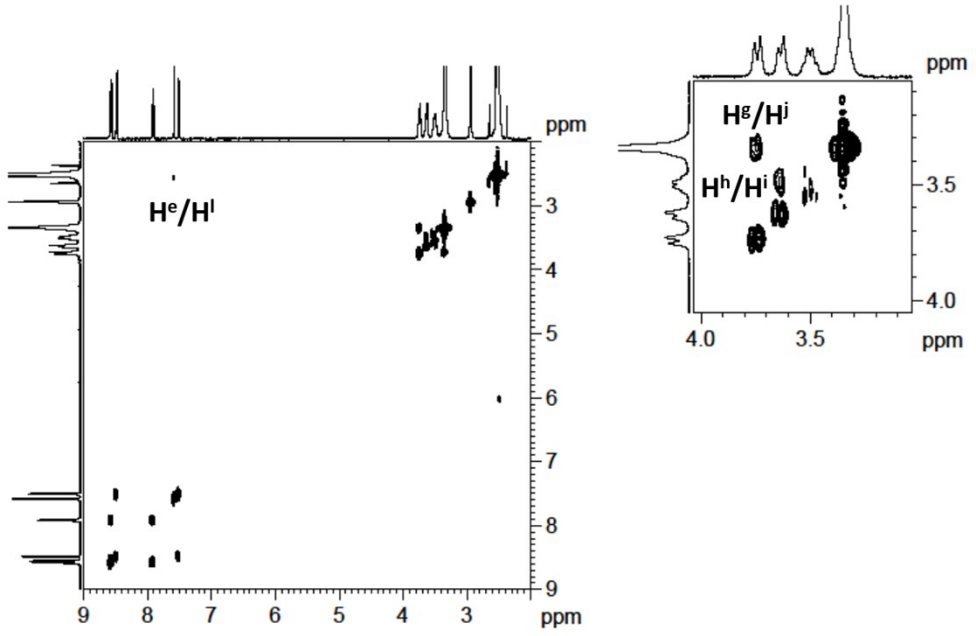

Figure S11. ^{1}H-^{1}H COSY NMR spectrum (500 MHz, 298.5K, DMSO-d_6) of [**TNP**H]$^{+}$.

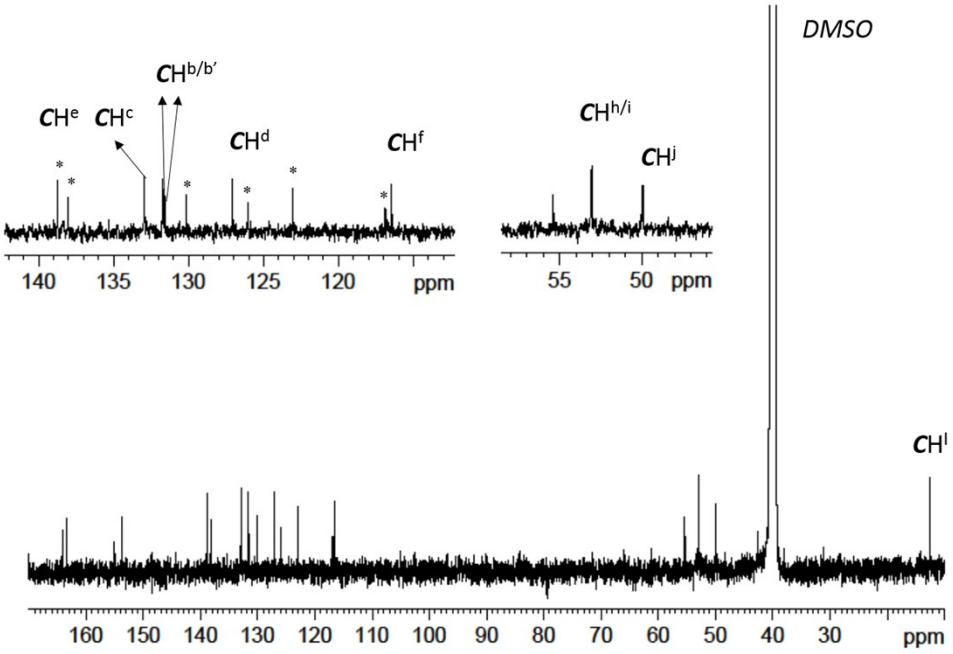

Figure S12. ^{13}C NMR (125 MHz, 298.5K, DMSO-d_6) of [**TNP**H]$^{+\cdot}$. * refer to the aromatic resonances of the naphthalimide and thiazol rings.

10. Single crystal X-ray diffraction

Single crystal structure of aqueous TNP·HCl resulting from TNP in presence of excess of $FeCl_3$ and aqueous DMSO.

Selected bond lengths /A ˚ and angles /°: N1-H1 0.89(2), O3 H3D 0.88(3)

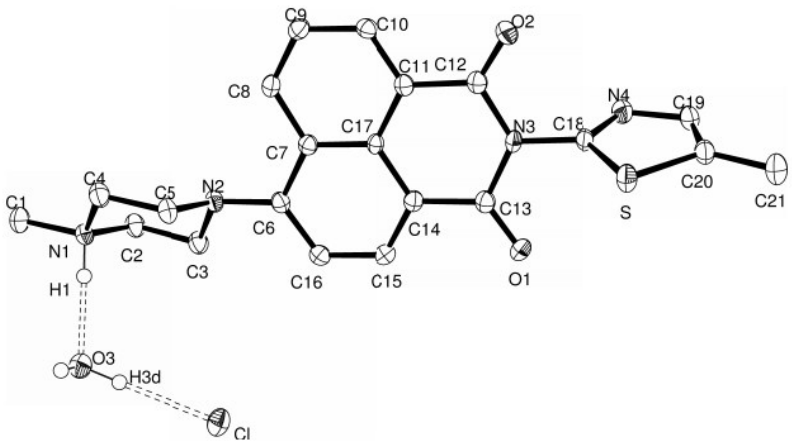

Figure S13. Molecular structure of **TNP**·HCl. CCDC deposition number:1542385.

Table 1. Crystal data and structure refinement for k14sip1.

Identification code	k14sip1	
Empirical formula	C21 H23 Cl N4 O3 S	
Formula weight	446.94	
Temperature	150(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P -1	
Unit cell dimensions	a = 7.2813(2) Å	α= 91.1888(12)°.
	b = 9.4897(3) Å	β= 101.8010(12)°.
	c = 15.4934(6) Å	γ= 100.7680(15)°.
Volume	1027.54(6) $Å^3$	
Z	2	

Density (calculated)	1.445 Mg/m^3
Absorption coefficient	0.320 mm^{-1}
F(000)	468
Crystal size	0.500 x 0.200 x 0.100 mm^3
Theta range for data collection	2.914 to 27.482°.
Index ranges	-9<=h<=9, -12<=k<=12, -20<=l<=20
Reflections collected	15108
Independent reflections	4689 [R(int) = 0.0688]
Completeness to theta = 25.242°	99.7 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.984 and 0.910
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4689 / 0 / 285
Goodness-of-fit on F^2	1.032
Final R indices [I>2sigma(I)]	R1 = 0.0406, wR2 = 0.0890
R indices (all data)	R1 = 0.0629, wR2 = 0.0970
Extinction coefficient	n/a
Largest diff. peak and hole	0.292 and -0.282 $e.Å^{-3}$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for **TNP**HCl. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
Cl	2815(1)	-2488(1)	4582(1)	30(1)
S	4354(1)	-4860(1)	-1600(1)	24(1)
N(1)	2031(2)	2399(2)	4071(1)	20(1)
N(2)	1674(2)	731(1)	2456(1)	19(1)
N(3)	2396(2)	-3003(1)	-959(1)	18(1)
N(4)	969(2)	-4479(2)	-2278(1)	23(1)
O(1)	2048(2)	-4763(1)	0(1)	24(1)
O(2)	3117(2)	-1239(1)	-1881(1)	22(1)
O(3)	3162(2)	618(2)	5372(1)	27(1)
C(1)	1788(3)	3670(2)	4589(1)	30(1)
C(2)	152(2)	1544(2)	3580(1)	22(1)
C(3)	473(2)	245(2)	3088(1)	21(1)
C(4)	3327(3)	2830(2)	3451(1)	24(1)
C(5)	3560(2)	1525(2)	2935(1)	22(1)

C(6)	1800(2)	-332(2)	1823(1)	18(1)
C(7)	2166(2)	122(2)	990(1)	17(1)
C(8)	2348(2)	1578(2)	764(1)	18(1)
C(9)	2720(2)	1983(2)	-37(1)	19(1)
C(10)	2880(2)	949(2)	-665(1)	18(1)
C(11)	2647(2)	-481(2)	-483(1)	17(1)
C(12)	2750(2)	-1544(2)	-1170(1)	18(1)
C(13)	2149(2)	-3492(2)	-126(1)	18(1)
C(14)	2037(2)	-2394(2)	535(1)	18(1)
C(15)	1648(2)	-2801(2)	1332(1)	21(1)
C(16)	1531(3)	-1777(2)	1974(1)	22(1)
C(17)	2296(2)	-916(2)	342(1)	16(1)
C(18)	2400(2)	-4058(2)	-1625(1)	18(1)
C(19)	1403(3)	-5509(2)	-2810(1)	24(1)
C(20)	3147(3)	-5865(2)	-2556(1)	23(1)
C(21)	3972(3)	-6960(2)	-2985(1)	33(1)

Table 3. Bond lengths [Å] for k14sip1.

S-C(20)	1.7235(18)
S-C(18)	1.7294(17)
N(1)-C(4)	1.491(2)
N(1)-C(1)	1.492(2)
N(1)-C(2)	1.497(2)
N(1)-H(1)	0.89(2)
N(2)-C(6)	1.421(2)
N(2)-C(3)	1.467(2)
N(2)-C(5)	1.475(2)
N(3)-C(13)	1.414(2)
N(3)-C(12)	1.418(2)
N(3)-C(18)	1.423(2)
N(4)-C(18)	1.291(2)
N(4)-C(19)	1.386(2)
O(1)-C(13)	1.216(2)
O(2)-C(12)	1.215(2)
O(3)-H(3C)	0.82(3)
O(3)-H(3D)	0.88(3)

C(2)-C(3)	1.516(2)
C(4)-C(5)	1.515(2)
C(6)-C(16)	1.381(2)
C(6)-C(7)	1.430(2)
C(7)-C(8)	1.421(2)
C(7)-C(17)	1.421(2)
C(8)-C(9)	1.372(2)
C(9)-C(10)	1.404(2)
C(10)-C(11)	1.378(2)
C(11)-C(17)	1.409(2)
C(11)-C(12)	1.474(2)
C(13)-C(14)	1.471(2)
C(14)-C(15)	1.371(2)
C(14)-C(17)	1.426(2)
C(15)-C(16)	1.402(2)
C(19)-C(20)	1.357(3)
C(20)-C(21)	1.501(2)

Table 4. Bond angles [°] for k14sip1.

C(20)-S-C(18)	89.28(8)
C(4)-N(1)-C(1)	111.53(14)
C(4)-N(1)-C(2)	110.33(14)
C(1)-N(1)-C(2)	111.99(14)
C(4)-N(1)-H(1)	110.6(15)
C(1)-N(1)-H(1)	105.9(14)
C(2)-N(1)-H(1)	106.2(14)
C(6)-N(2)-C(3)	115.99(13)
C(6)-N(2)-C(5)	113.14(13)
C(3)-N(2)-C(5)	109.75(14)
C(13)-N(3)-C(12)	125.43(14)
C(13)-N(3)-C(18)	117.27(13)
C(12)-N(3)-C(18)	117.20(14)
C(18)-N(4)-C(19)	109.33(15)
H(3C)-O(3)-H(3D)	108(3)
H(1B)-C(1)-H(1C)	109.5

N(1)-C(2)-C(3)	109.90(14)
N(2)-C(3)-C(2)	109.13(14)
N(1)-C(4)-C(5)	110.71(14)
N(1)-C(4)-H(4A)	109.5
N(2)-C(5)-C(4)	110.12(14)
C(16)-C(6)-N(2)	122.57(16)
C(16)-C(6)-C(7)	119.28(15)
N(2)-C(6)-C(7)	118.13(14)
C(8)-C(7)-C(17)	117.84(15)
C(8)-C(7)-C(6)	122.67(15)
C(17)-C(7)-C(6)	119.46(14)
C(9)-C(8)-C(7)	121.29(16)
C(8)-C(9)-C(10)	120.20(15)
C(11)-C(10)-C(9)	120.23(16)
C(10)-C(11)-C(17)	120.44(15)
C(10)-C(11)-C(12)	118.78(16)
C(17)-C(11)-C(12)	120.77(14)
O(2)-C(12)-N(3)	119.85(15)
O(2)-C(12)-C(11)	124.17(15)
N(3)-C(12)-C(11)	115.98(15)
O(1)-C(13)-N(3)	119.20(15)
O(1)-C(13)-C(14)	124.50(17)
N(3)-C(13)-C(14)	116.30(14)
C(15)-C(14)-C(17)	120.07(15)
C(15)-C(14)-C(13)	119.66(15)
C(17)-C(14)-C(13)	120.25(16)
C(14)-C(15)-C(16)	120.86(15)
C(6)-C(16)-C(15)	121.16(17)
C(11)-C(17)-C(7)	119.93(14)
C(11)-C(17)-C(14)	120.91(15)
C(7)-C(17)-C(14)	119.15(15)
N(4)-C(18)-N(3)	122.93(15)
N(4)-C(18)-S	115.75(13)
N(3)-C(18)-S	121.31(12)
C(20)-C(19)-N(4)	116.85(16)
C(19)-C(20)-C(21)	128.40(17)
C(19)-C(20)-S	108.79(13)
C(21)-C(20)-S	122.79(15)

Table 5. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for k14sip1. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + ...\ + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
Cl	32(1)	22(1)	34(1)	-3(1)	5(1)	1(1)
S	23(1)	24(1)	24(1)	-6(1)	3(1)	7(1)
N(1)	27(1)	20(1)	15(1)	-2(1)	4(1)	6(1)
N(2)	20(1)	19(1)	16(1)	-2(1)	5(1)	2(1)
N(3)	23(1)	14(1)	18(1)	-4(1)	5(1)	4(1)
N(4)	26(1)	22(1)	20(1)	-2(1)	1(1)	4(1)
O(1)	36(1)	16(1)	24(1)	1(1)	8(1)	8(1)
O(2)	24(1)	24(1)	18(1)	0(1)	7(1)	5(1)
O(3)	31(1)	25(1)	26(1)	-1(1)	5(1)	8(1)
C(1)	42(1)	24(1)	24(1)	-5(1)	8(1)	10(1)
C(2)	23(1)	25(1)	20(1)	-2(1)	7(1)	3(1)
C(3)	23(1)	22(1)	19(1)	-1(1)	7(1)	1(1)
C(4)	26(1)	23(1)	20(1)	-2(1)	7(1)	-1(1)
C(5)	20(1)	24(1)	20(1)	-3(1)	4(1)	2(1)
C(6)	17(1)	20(1)	16(1)	-1(1)	2(1)	4(1)
C(7)	15(1)	17(1)	17(1)	0(1)	2(1)	2(1)
C(8)	18(1)	16(1)	19(1)	-3(1)	2(1)	3(1)
C(9)	19(1)	16(1)	22(1)	1(1)	2(1)	2(1)
C(10)	18(1)	19(1)	16(1)	2(1)	4(1)	2(1)
C(11)	14(1)	18(1)	17(1)	-1(1)	1(1)	3(1)
C(12)	14(1)	19(1)	18(1)	-1(1)	1(1)	3(1)
C(13)	16(1)	19(1)	19(1)	-2(1)	2(1)	4(1)
C(14)	17(1)	17(1)	19(1)	-1(1)	2(1)	4(1)
C(15)	25(1)	17(1)	22(1)	2(1)	5(1)	5(1)
C(16)	29(1)	21(1)	17(1)	2(1)	7(1)	6(1)
C(17)	14(1)	15(1)	16(1)	-2(1)	2(1)	2(1)
C(18)	22(1)	16(1)	18(1)	-2(1)	6(1)	3(1)
C(19)	32(1)	21(1)	17(1)	-4(1)	3(1)	3(1)
C(20)	30(1)	18(1)	19(1)	-2(1)	8(1)	2(1)
C(21)	44(1)	30(1)	28(1)	-6(1)	12(1)	12(1)

Table 6. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for k14sip1.

	x	y	z	U(eq)
H(1)	2520(30)	1830(20)	4468(16)	40(6)
H(3C)	4310(40)	980(30)	5511(19)	59(8)
H(3D)	3060(40)	-260(30)	5158(19)	69(9)
H(1A)	3032	4146	4944	44
H(1B)	908	3353	4979	44
H(1C)	1265	4345	4183	44
H(2A)	-503	2151	3156	27
H(2B)	-675	1226	4001	27
H(3A)	1109	-372	3511	26
H(3B)	-771	-327	2770	26
H(4A)	4593	3332	3789	28
H(4B)	2792	3503	3037	28
H(5A)	4378	1833	2509	26
H(5B)	4194	889	3344	26
H(8)	2210	2285	1177	22
H(9)	2870	2966	-168	23
H(10)	3150	1236	-1217	21
H(15)	1455	-3790	1452	25
H(16)	1262	-2083	2523	26
H(19)	522	-5945	-3327	29
H(21A)	2991	-7499	-3468	49
H(21B)	4406	-7623	-2548	49
H(21C)	5058	-6473	-3221	49

Table 7. Hydrogen bonds for k14sip1 [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
N(1)-H(1)...O(3)	0.89(2)	1.87(2)	2.754(2)	170(2)
O(3)-H(3C)...Cl#1	0.82(3)	2.34(3)	3.1147(16)	158(3)
O(3)-H(3D)...Cl	0.88(3)	2.24(3)	3.1130(15)	179(3)

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,-y,-z+1

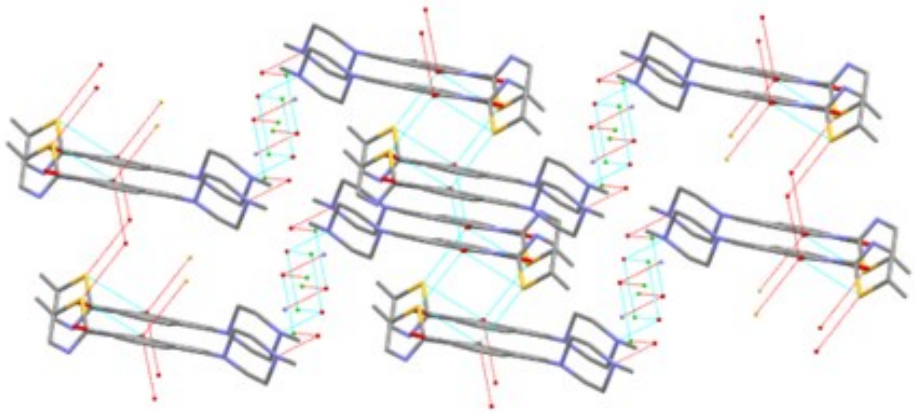

Figure 14. Fragment of the unit cell, view over axis b. Image shows extended H-bonds involving the Cl- ions and the H_2O molecules. Crystals were grown from a mixture of 2:1 TNP and $FeCl_3$ in wet DMSO. Colour code: Yellow: Sulfur, Red: Oxygen, Grey: Carbon, Light grey: Hydrogen, Green: Chlorine. Details are given in the ESI and Figure S12)

11. Cell culture and imaging experiments setup

(HeLa, PC3 and CHO) cells were grown as monolayers in T75 tissue culture flasks, and cultured in Eagle's Minimum Essential Medium (EMEM) for Hela, Roswell Park Memorial Institute medium (RPMI 1640) for PC-3, supplemented with 10% foetal bovine serum, 1% L-glutamine (200 mM), 0.5% penicillin/streptomycin (10 000 IU mL^{-1}/10 000 mg mL^{-1}). Cells were maintained at 37 ^{o}C in a 5% CO_2 humidified atmosphere and grown to approximately 85% confluence before being split using a 2.5% trypsin solution. For microscopy, cells were seeded into glass bottomed Petri dishes and incubated for 12 h for HeLa and 24 h for PC-3 to ensure adhesion. Cells were plated in 35 mm uncoated 1.5 mm thick glass-bottomed dishes as 3 × 105 cells per dish and incubated for at least 24 h prior to imaging experiment. **TNP** complexes were prepared as 5 mM solutions in DMSO and diluted to 50 μM with serum free EMEM. Cells were washed 5 times with 1 mL Hank's Balanced Salt Solution (HBSS) and incubated with the 50 μm (1% DMSO) or 100 μm (2% DMSO) of **TNP** compounds at 37 ^{o}C for 15 min. Background autofluorescence was measured by imaging the cells in 1 mL of serum free EMEM only. Immediately prior to imaging cells were washed 3 times with 1 mL HBSS and returned to serum free EMEM.

Imaging experiments

The fluorescent uptake of the **TNP** complex was imaged by laser-scanning confocal microscopy. Initial experiments for cells viability and uptake were recorded using a using a Zeiss LSM 510 META microscope irradiating at 488 nm with emission filtered between 505 and 535 nm. 5 eq. of $FeCl_3$ was consequently added to the serum free medium; cells were incubated 15 minutes and washed three times with HBSS prior to imaging. The same experiment was repeated at the OCTOPUS facility at the Research Complex at Harwell, using single photon and 2-photon laser irradiation with the specifications below.

Two-photon (690-1000 nm) wavelength laser light was obtained from the mode-locked titanium- sapphire laser Mira (Coherent Laser Co., Ltd.) produced by 180 femtosecond pulse frequency of 75 MHz. This laser-pumped solid-state continuous

wave 532 nm laser (Verdi V18, Coherent Laser Co., Ltd.). This canalso be used for the fundamental output of the oscillator 915 ± 2 nm. The laser beam was focused to a diffraction-limited spot by the water immersion UV calibration target (Nikon VC × 60, NA1.2) and the specimen on a microscope stage of the modified Nikon TE2000-U, with UV illumination optical emission. The focused laser beam raster scanning used an X - Y galvanometer (GSI Lumonics Corporation). Fluorescence emission was collected without de-scanning, bypassing the scanning system and passed through a coloured glass (BG39) filter. In normal operation mode and line scan frame and pixel clock signal was generated with an external fast microchannel plate photomultiplier tube as detector (R3809 - U, Hamamatsu, Japan) synchronisation. These were linked to a Time Correlated Single Photon Counting (TCSPC) PC module SPC830 for the lifetime measurements with 915 nm excitation and emission in the range between 360 and 580 nm.

12. Confocal and 2-photon fluorescence imaging

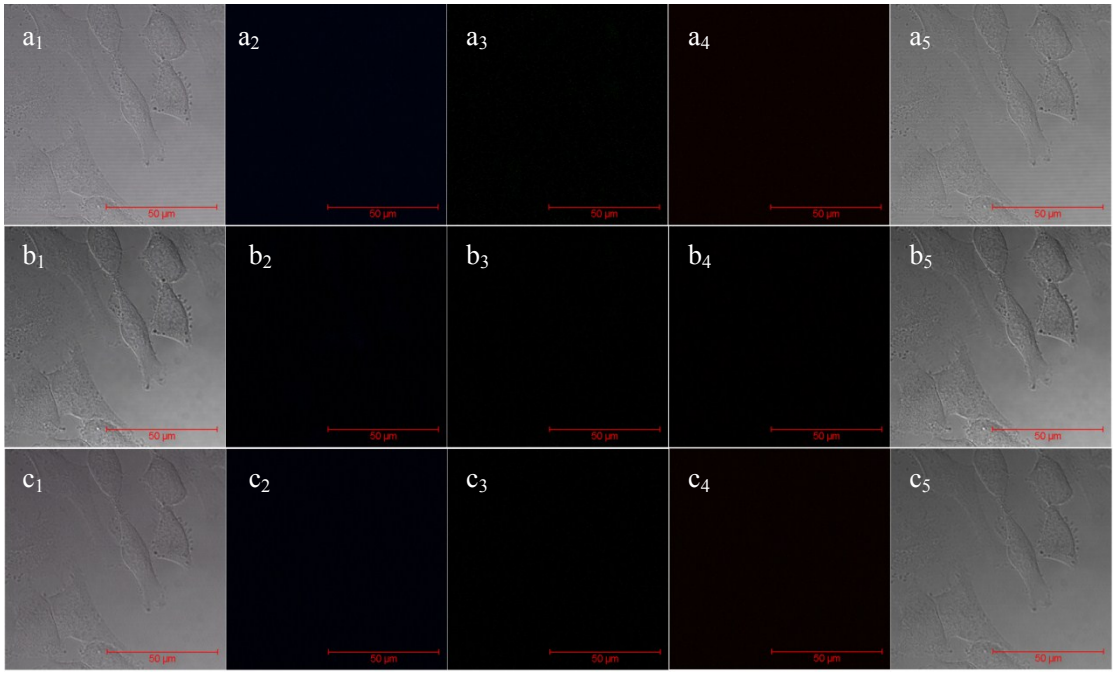

Figure S15. Confocal fluorescence imaging of cervical cancer (HeLa) showing cell control, λ_{ex}= 405 nm, 488 nm and 543 nm, λ_{em}= 450 nm, 515 nm and 605 nm, respectively. a_1-a_4) λ_{ex} = 405.0 nm; b_1-b_4) λ_{ex} = 488.0 nm; c_1-c_4) λ_{ex} = 561.0 nm. a_1-b_1-c_1 overlay of the blue-green-red channels; a_2-b_2-c_2) blue channel (λ_{em} = 417-477 nm); a_3-b_3-c_3) green channel (λ_{em} = 500-550 nm); a_4-b_4-c_4) red channel (λ_{em} = 570-750 nm). a_5-b_5-c_5) DIC channel. Scale bar: 50 µm.

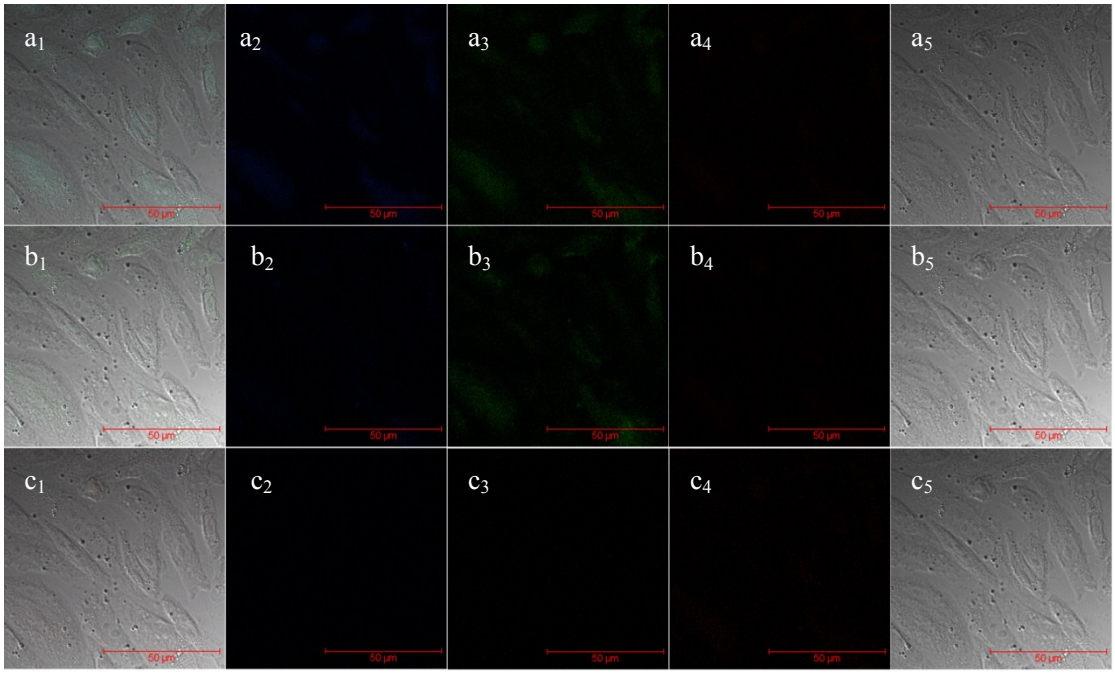

Figure S16. Confocal fluorescence imaging of prostate cancer (PC-3) cells as the control, λ_{ex}= 405 nm, 488 nm and 543 nm, λ_{em}= 450 nm, 515 nm and 605 nm, respectively. a_1-a_4) λ_{ex} = 405.0 nm; b_1-b_4) λ_{ex} = 488.0 nm; c_1-c_4) λ_{ex} = 561.0 nm. a_1-b_1-c_1 overlay of the blue-green-red channels; a_2-b_2-c_2) blue channel (λ_{em} = 417-477 nm); a_3-b_3-c_3) green channel (λ_{em} = 500-550 nm); a_4-b_4-c_4) red channel (λ_{em} = 570-750 nm). a_5-b_5-c_5) DIC channel. Scale bar: 50 μm.

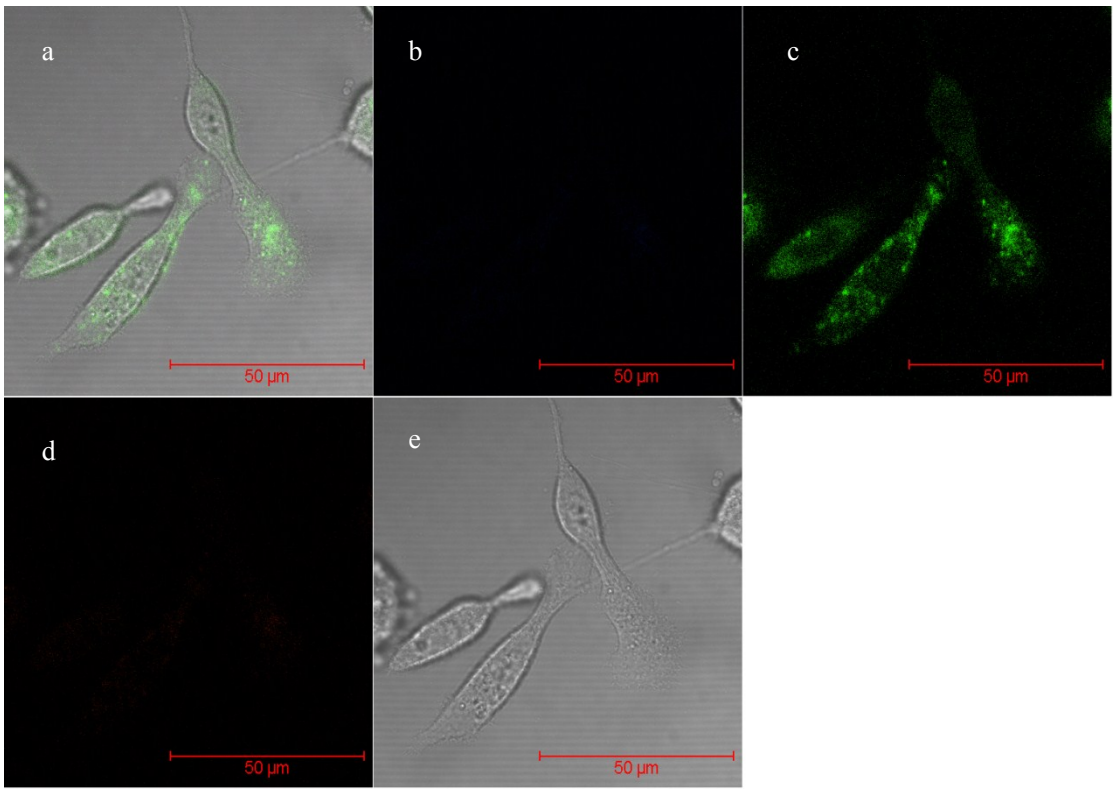

Figure S17. Confocal fluorescence imaging of PC-3 cells (37 ^{o}C, 15 minutes incubation with the addition of 100 µM of **TNP**, 5 eq $FeCl_3$, 2% DMSO, λ_{ex}= 488 nm, λ_{em}= 450 nm, 515 nm and 605 nm, respectively). (a) Represents the overlaid image of (b)-(e) micrographs respectively. The (b) image show cells excited at 488 nm where emission was collected in the 'blue channel' up to 450 nm; (c) is an image obtained under emission wavelength at 515 nm); (d) is an image obtained under emission wavelength at 605 nm. The micrograph (e) is the corresponding DIC image.

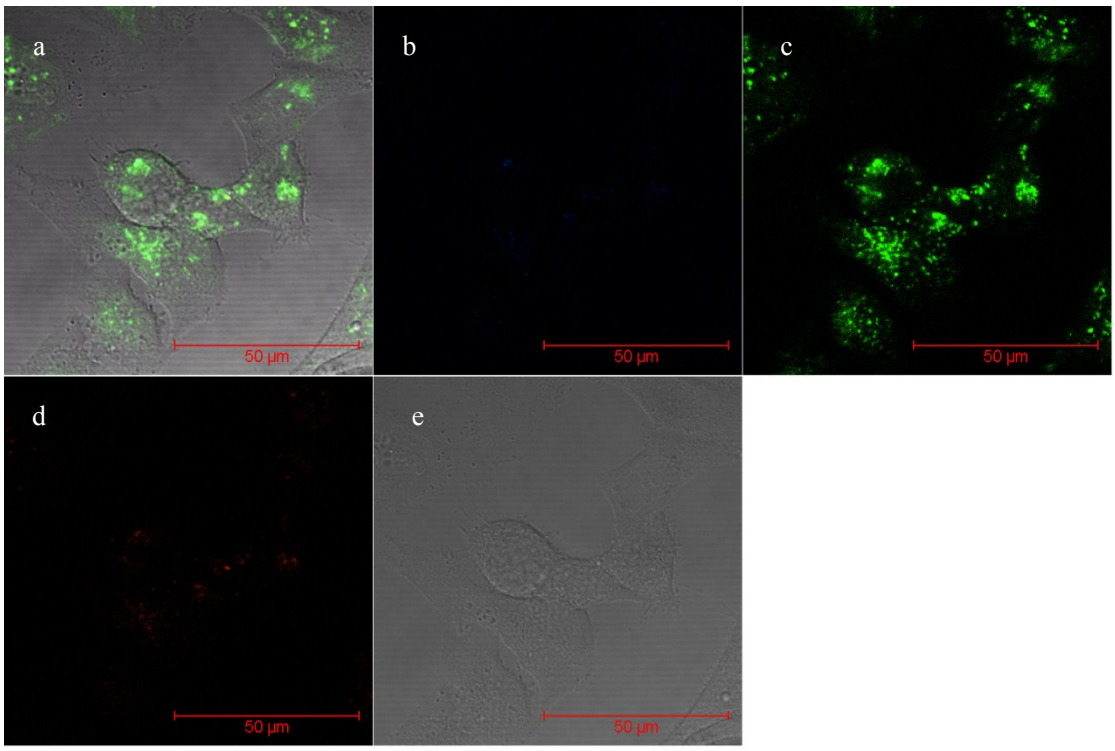

Figure S18. Confocal fluorescence imaging of HeLa cells (37 ^{o}C, 15 minutes incubation with the addition of 50 µM, 1% DMSO of **TNP** containing 5 eq. of $FeCl_3$, λ_{ex}= 488 nm, λ_{em}= 450 nm, 515 nm and 605 nm, respectively). (a) Represents the overlaid image of (b)-(e) micrographs respectively. The (b) image show cells excited at 488 nm where emission was collected in the 'blue channel' up to 450 nm; (c), is an image obtained under emission wavelength at 515 nm); (d) is an image obtained under emission wavelength at 605 nm. The micrograph (e) is the corresponding DIC image.

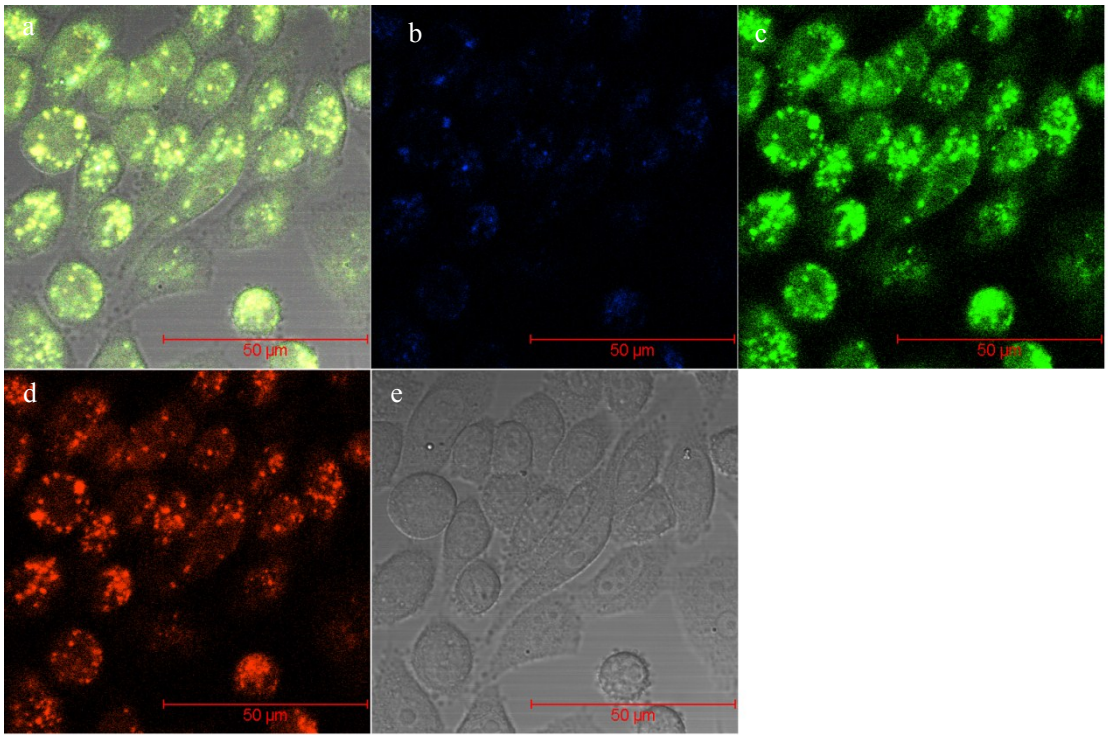

Figure S19. Confocal fluorescence imaging of [**TNP**H]Cl in CHO cells (37 ^{o}C, 15 minutes incubation with the addition of 50 µM of compound, λ_{ex}= 488 nm, λ_{em}= 450 nm, 515 nm and 605 nm, respectively). (a) Represents the overlaid image of (b)-(e) micrographs respectively. The (b) image show cells excited at 488 nm where emission was collected in the 'blue channel' (λ_{em} = 417-477 nm); (c), is an image obtained under emission wavelength at 515 nm); (d) is image obtained under emission wavelength at 605 nm. The micrograph (e) is the corresponding DIC image.

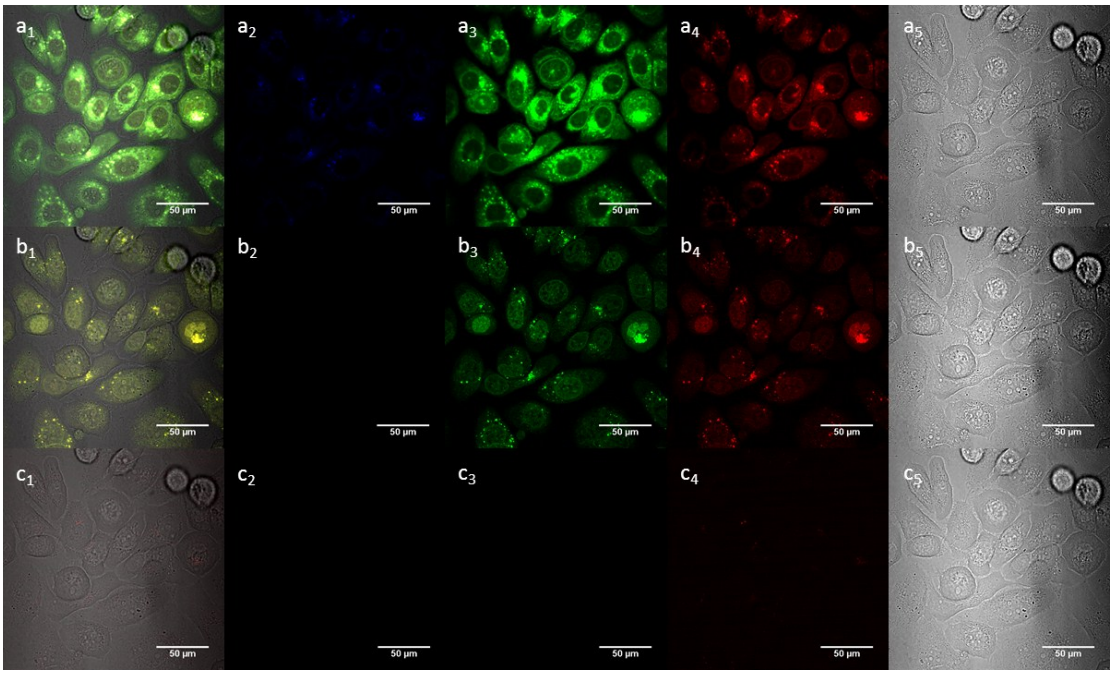

Figure S20. Confocal fluorescence imaging of [**TNP**H]Cl in PC3 cells (37 ^{o}C, 15 minutes incubation with the addition of 10 mM of compound 1:99 in cell media, λ_{ex}= 405, 488, 561 nm). a_1-a_4) λ_{ex} = 405.0 nm; b_1-b_4) λ_{ex} = 488.0 nm; c_1-c_4) λ_{ex} = 561.0 nm. a_1-b_1-c_1 overlay of the blue-green-red channels; a_2-b_2-c_2) blue channel (λ_{em} = 417-477 nm); a_3-b_3-c_3) green channel (λ_{em} = 500-550 nm); a_4-b_4-c_4) red channel (λ_{em} = 570-750 nm). a_5-b_5-c_5) DIC channel. Scale bar: 50 µm.

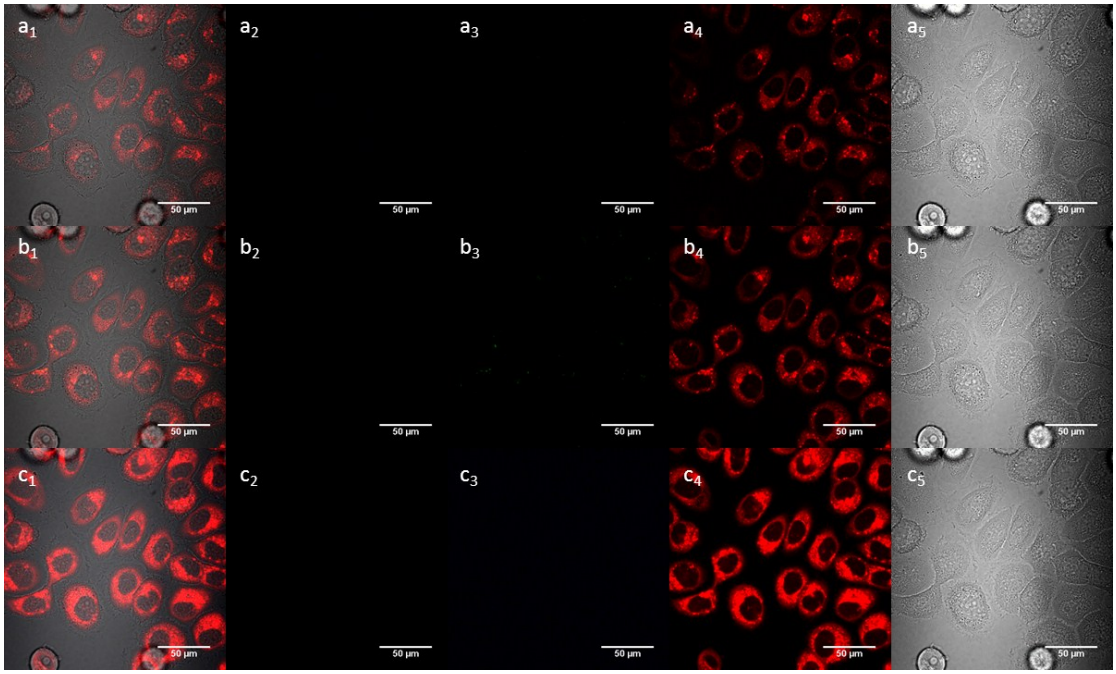

Figure S21. Confocal fluorescence imaging of lysosome red tracker in PC3 cells (37 ^{o}C, 30 minutes incubation with the addition of 25 µM of dye 1:99 in cell media, λ_{ex}= 405, 488, 561 nm. a_1-a_4) λ_{ex} = 405.0 nm; b_1-b_4) λ_{ex} = 488.0 nm; c_1-c_4) λ_{ex} = 561.0 nm. a_1-b_1-c_1 overlay of the blue-green-red channels; a_2-b_2-c_2) blue channel (λ_{em} = 417-477 nm); a_3-b_3-c_3) green channel (λ_{em} = 500-550 nm); a_4-b_4-c_4) red channel (λ_{em} = 570-750 nm). a_5-b_5-c_5) DIC channel. Scale bar: 50 µm.

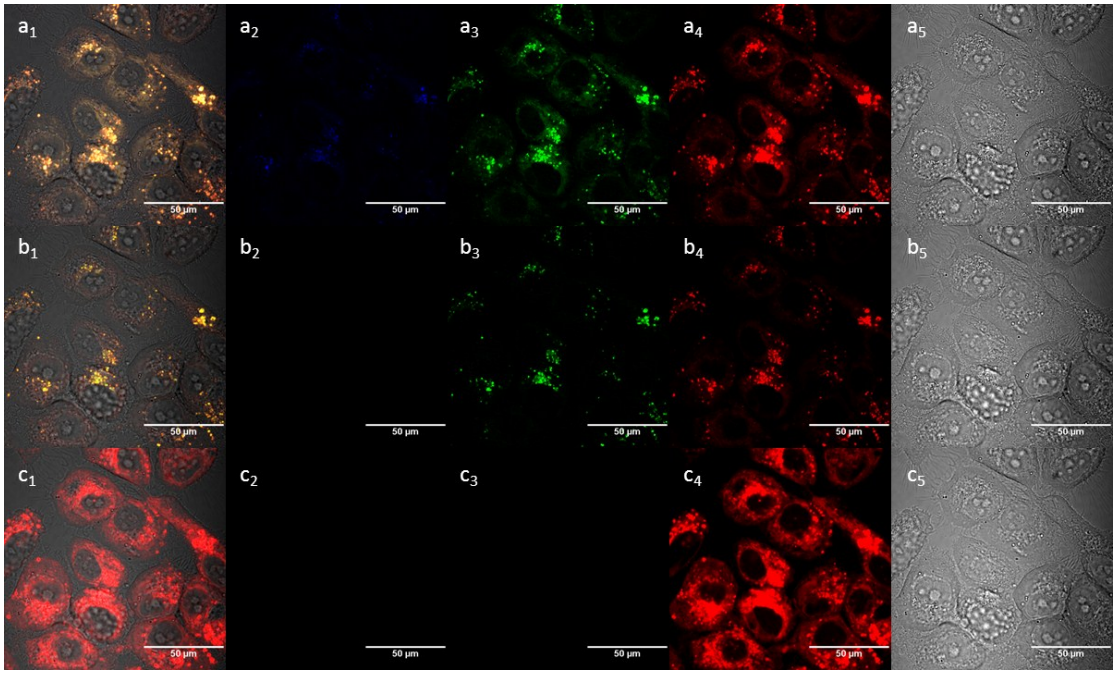

Figure S22. Confocal fluorescence imaging of [**TNP**H]Cl in PC3 cells (37 ºC, 15 minutes incubation with the addition of 10 mM of compound 1:99 in cell media, λ_{ex}= 405, 488, 561 nm, cells were pre-incubated with lysosome red tracker (25 µM lysosome tracker 1:99 in cell media, 30 minutes incubation at 37 ºC). a_1-a_4) λ_{ex} = 405.0 nm; b_1-b_4) λ_{ex} = 488.0 nm; c_1-c_4) λ_{ex} = 561.0 nm. a_1-b_1-c_1 overlay of the blue-green-red channels; a_2-b_2-c_2) blue channel (λ_{em} = 417-477 nm); a_3-b_3-c_3) green channel (λ_{em} = 500-550 nm); a_4-b_4-c_4) red channel (λ_{em} = 570-750 nm). a_5-b_5-c_5) DIC channel. Scale bar: 50 µm.

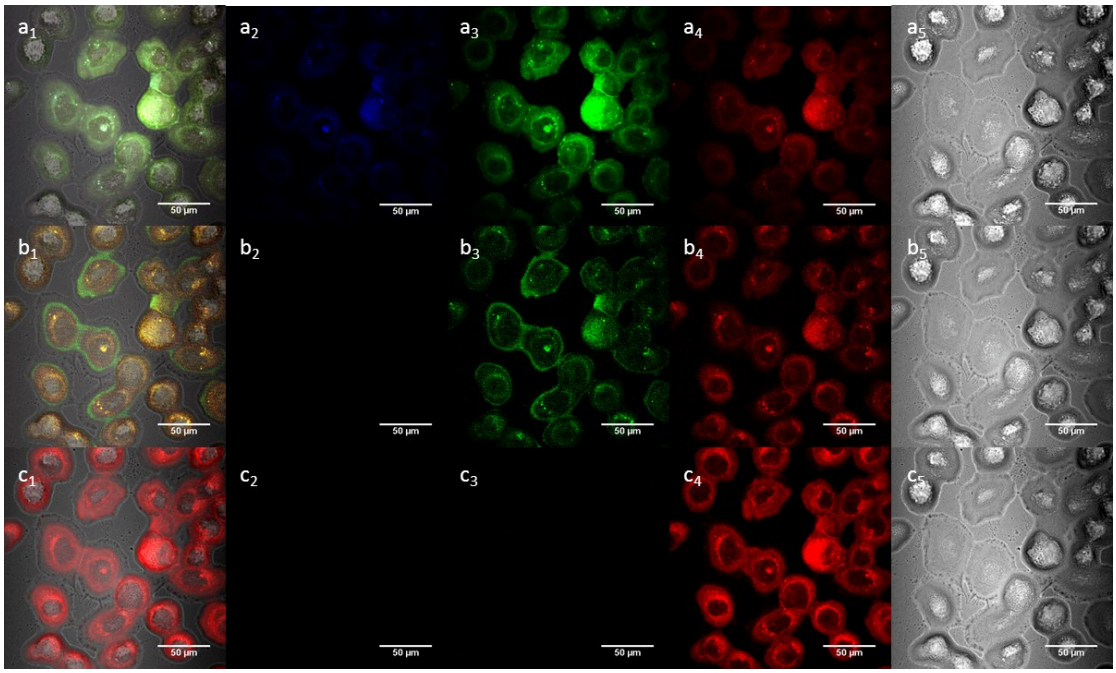

Figure S23. Confocal fluorescence imaging of [**TNP**H]$^+$ in PC3 cells (37 ^{o}C, 15 minutes incubation with the addition of 10 mM of compound 1:99 in cell media, λ_{ex}= 405, 488, 561 nm, cells were pre-incubated with lysosome red tracker (25 µM lysosome tracker 1:99 in cell media, 30 minutes incubation at 37 ^{o}C). Cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes. a_1-a_4) λ_{ex} = 405.0 nm; b_1-b_4) λ_{ex} = 488.0 nm; c_1-c_4) λ_{ex} = 561.0 nm. a_1-b_1-c_1 overlay of the blue-green-red channels; a_2-b_2-c_2) blue channel (λ_{em} = 417-477 nm); a_3-b_3-c_3) green channel (λ_{em} = 500-550 nm); a_4-b_4-c_4) red channel (λ_{em} = 570-750 nm). a_5-b_5-c_5) DIC channel. Scale bar: 50 µm. However, our in vitro studies suggest that the lipophilic cation [**TNP**H]$^+$ permeates the PC-3 cells and localises into the lysosomes, where the acidic environment stabilises the formation of [**TNP**H$^+$]Cl$^-$, which can be visualised in the lysosomes by multiphoton FLIM.;

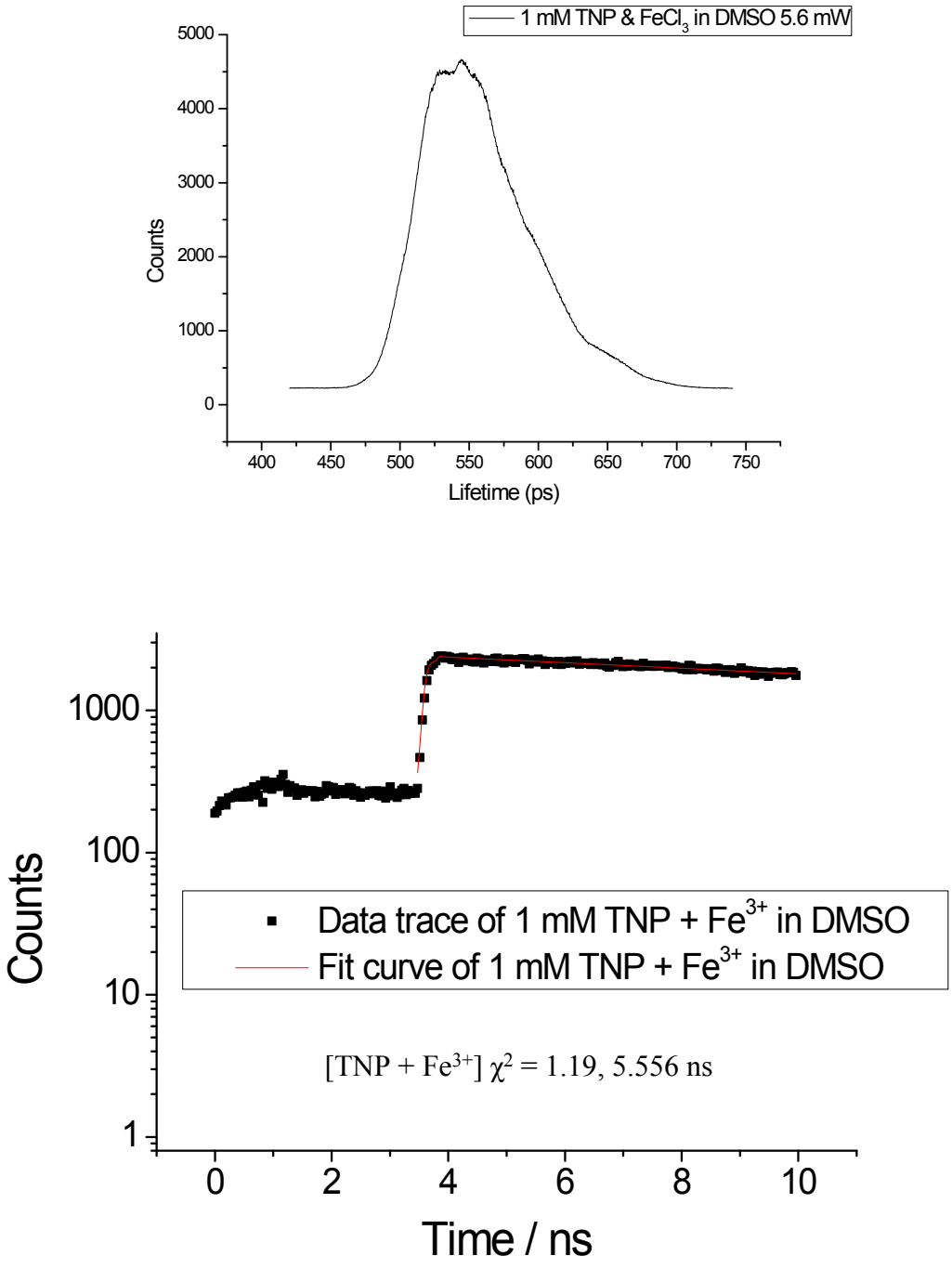

Figure S24. Two-photon spectroscopy of solution of **TNP** (1 mM **TNP** protonated in wet DMSO) in the presence of 5 eq. $FeCl_3$; top: emission spectrum, bottom: corresponding fluorescence lifetime spectrum).

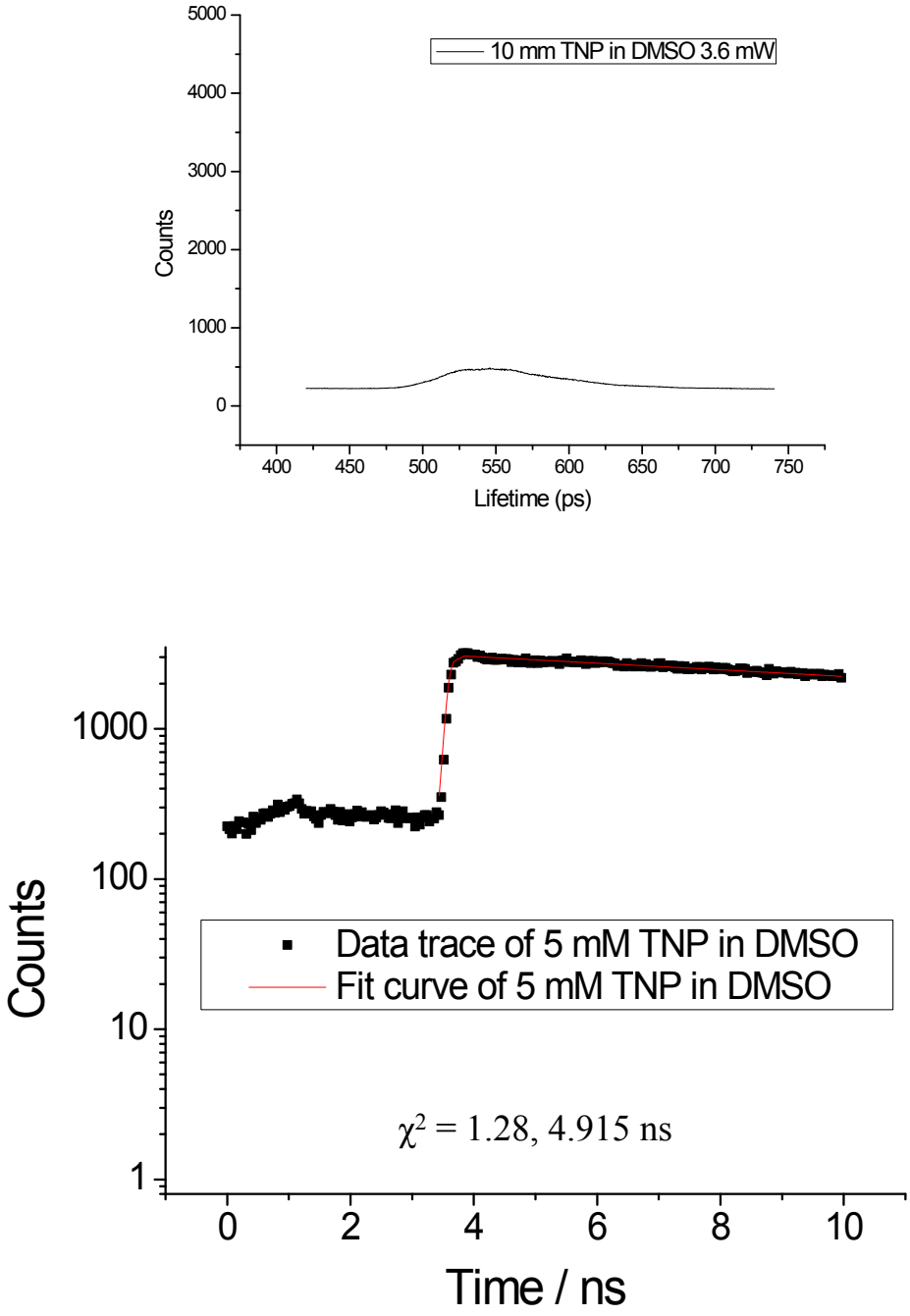

Figure S25. Two-photon spectroscopy of free **TNP** (10 mM in DMSO). Data only showed extremely weak, broad emission under 810 nm excitation. An emission spectrum could not be obtained below 5 mM conc. in absence of iron ions (top spectrum: 2P emission of free **TNP**; bottom: corresponding fluorescence lifetime with a reliable exponential fitting could be obtained from 5 mM conc. Solutions)

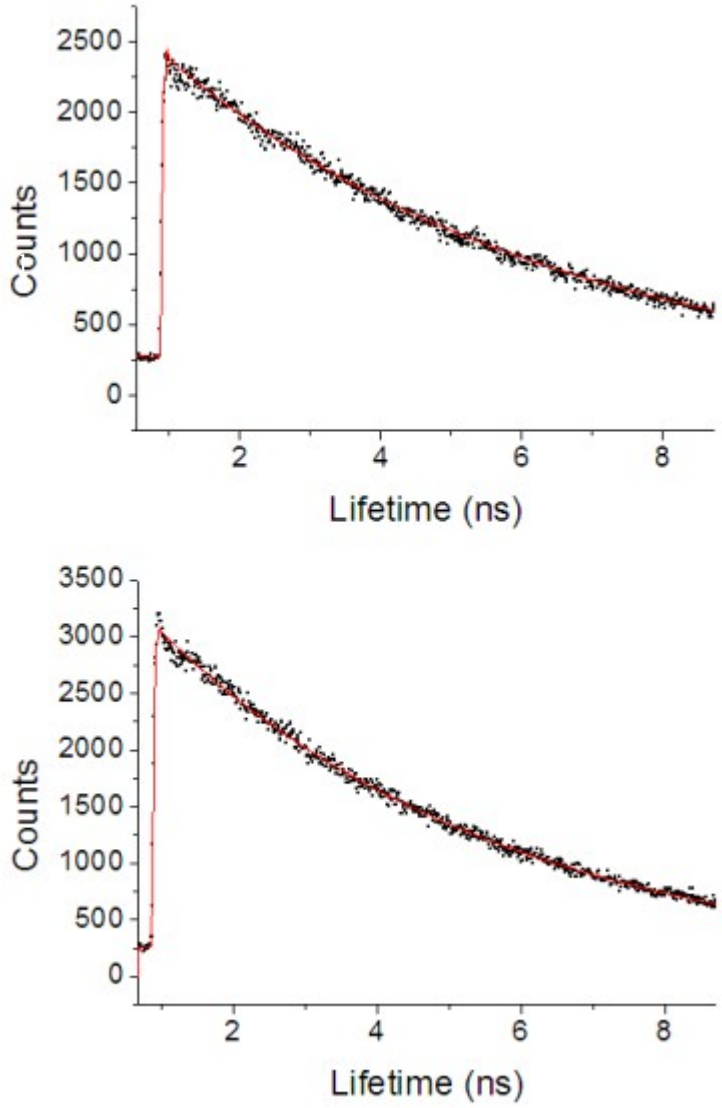

Figure S26. Solution TCSPC: Fluorescence lifetime (point-decay data) showing solution behavior in 10 mM solutions of **TNP** in aqueous DMSO, in the absence (top) and presence (bottom) of 5 eq. aqueous $FeCl_3$: 2-Photon fluorescence spectroscopy inclusive of data fitting.

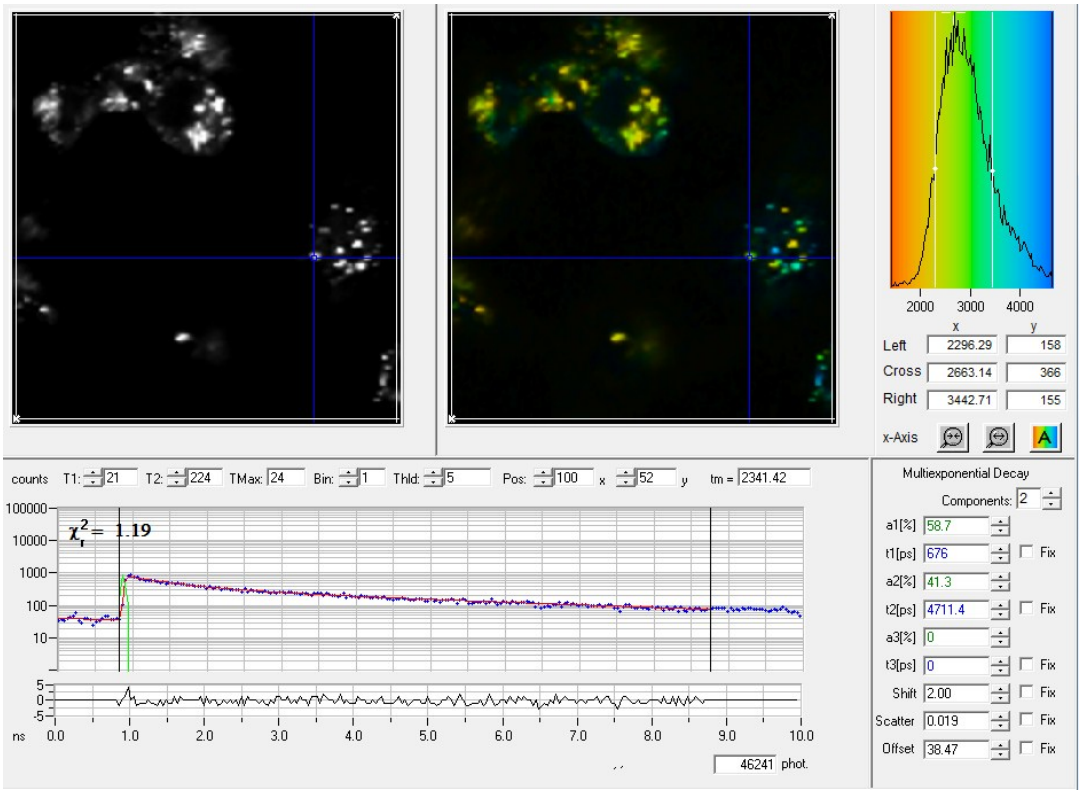

Figure S27. Two-photon imaging for **TNP** in the presence of 5 eq. $FeCl_3$ in HeLa cells (1% DMSO, 50 μM, 8.8 mW, 810 nm, 15 min incubation) Images show: 2P Cellular fluorescence intensity (left image), corresponding fluorescence lifetime imaging (middle image), and FLIM lifetime distribution map (right image). Multiexponential decay and corresponding fitting for lifetime in a set point inside the cells shows consistency with 2P point decay lifetime in solution for TNP in the presence of 5eq $FeCl_3$ above. The levels of intracellular iron(III) available for coordination are most likely very low to ensure a detectable concentration of the $[(\mathbf{TNP})_2FeCl_2]^+$ complex. Furthermore, such iron complex is not stable in aqueous environments and leads to the formation of the protonated species, $[\mathbf{TNP}H]^+$, and a large increase in fluorescence emission.

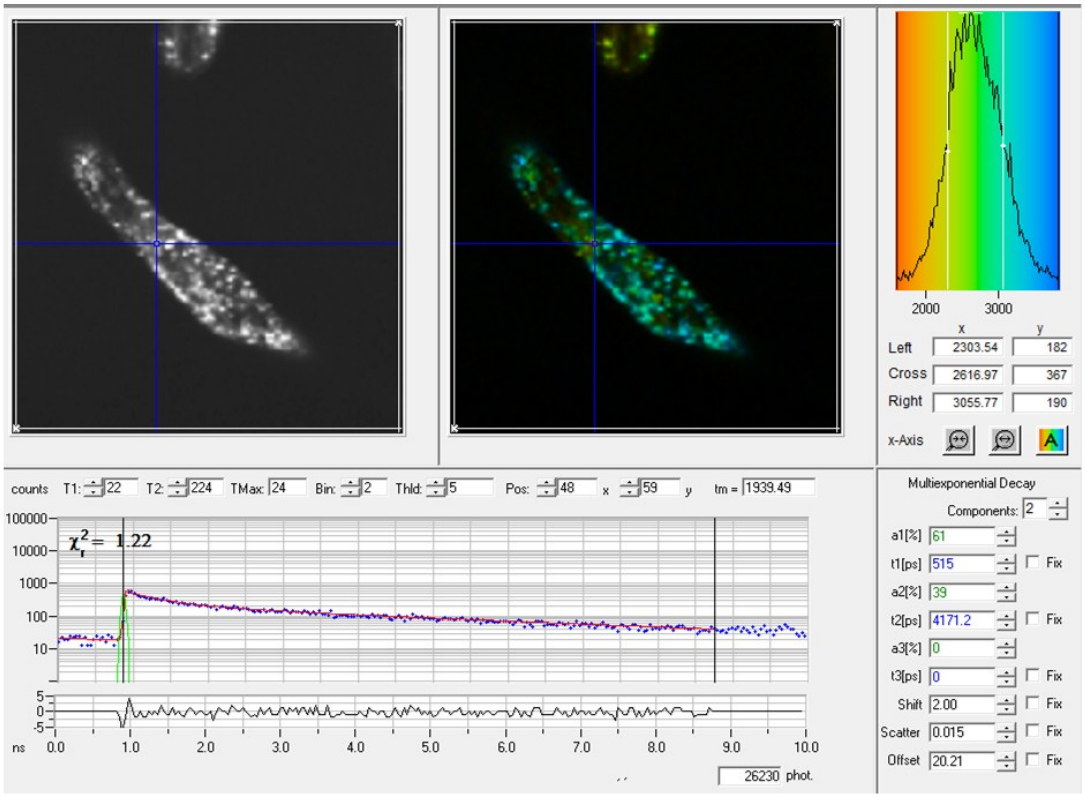

Figure S28. Two-photon imaging of **TNP** in the presence of $FeCl_3$ in PC-3 cells (2% DMSO, 100 μM, 8.8 mW, 810 nm, 15 min incubation). Images show: 2P Cellular fluorescence intensity (left image), corresponding fluorescence lifetime imaging (middle image), and lifetime distribution map (right image). Multiexponential decay and corresponding fitting for lifetime in a set point inside the cells shows consistency with 2P point decay lifetime in solution for **TNP** in the presence of 5eq $FeCl_3$.

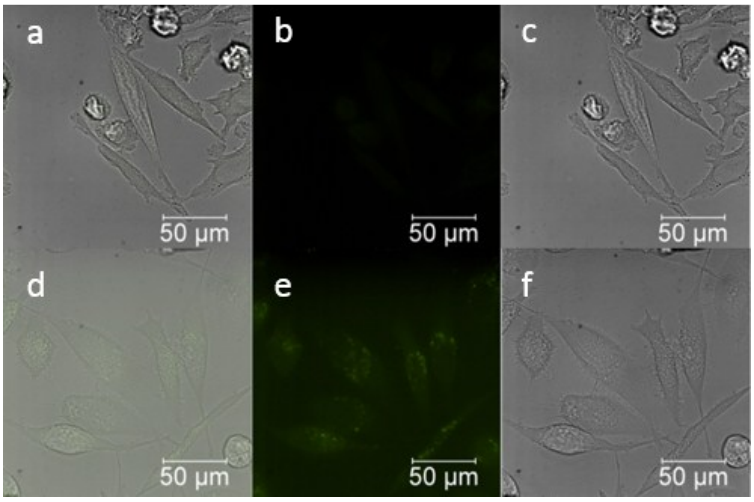

Figure S29. Confocal fluorescence images of PC-3 cells incubated at 37 °C for 15 minutes with TNP (a-c) and TNP protonated in the presence of aq $FeCl_3$, to form [TNPH]Cl (d-f) (50 µM, in 5 : 95 DMSO : serum free medium). (a, d) overlay of DIC and green channel; (b, e) green channel (λ_{ex} = 405 nm, (λ_{em} = 515 nm); (c, f) DIC channel.

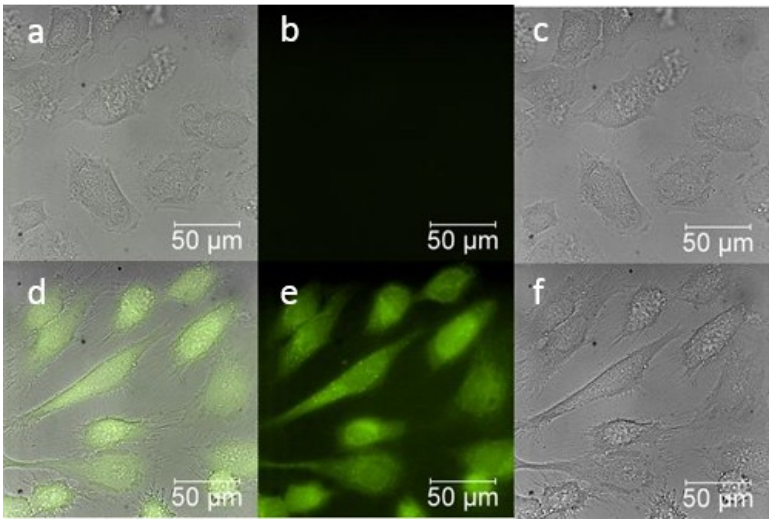

Figure S30. Epifluorescence imaging of HeLa cells incubated at 37 °C for 15 minutes with **TNP** (**a-c**), and **[TNP**H**]**$^+$ (formed in situ from TNP in the presence of aqueous media enriched with $FeCl_3$ **(d-f)** (50 µM, in 5 : 95 DMSO : serum free medium) (a, d): overlay of DIC and green channel; (b,e) fluorescent images excitation wavelength 460-500 nm with a long pass filter at 510 nm, (c,f) bright-field images.

13. Cell culture and cytotoxicity experiments setup

Cells were cultured at 37 °C in a humidified atmosphere in air and harvested once >70% confluence had been reached. EMT6 (breast cancer cells) were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium. The media contained 10% foetal calf serum (FCS), 0.5% penicillin/streptomycin (10,000 IU mL-1/10,000 mg mL-1) and 1% 200 mM L-Glutamine. All steps were performed in absence of phenol red. Supernatant containing dead cell matter and excess protein was aspirated. The live adherent cells were then washed with 10 mL of phosphate buffer saline (PBS) solution twice to remove any remaining media containing FCS, which may inactivate trypsin. Cells were incubated in 3 mL of trypsin solution (0.25% trypsin) for 5 to 7 min at 37 °C. After trypsinisation, 6 mL of medium containing 10% serum was added to inactivate the trypsin and the solution was centrifuged for 5 min (1000 rpm, 25 ºC) to remove any remaining dead cell matter. The supernatant liquid was aspirated and 5 mL of cell medium (10% FCS) was added to the cell matter left behind. Cells were counted using a haemocytometer and then seeded as appropriate.

Crystal violet cytotoxicity assays:

$6.3x10^6$ EMT6 cells were harvested and seed on nine 96 well plates (7000 EMT6 cells in each well), cells were incubated 24 hours for cell attachment. 8 different concentrations of **[TNPH]Cl** were loaded in 96 well plates (100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.1 μM, 1 nM). Each concentration repeated twice in a 96 well plates. 3x3 96 well plates were incubated for 24 hours, 48 hours and 72 hours respectively. After incubation, 96 wells plates were washed with PBS three times and 100 μL of methanol: PBS mixture (1:1) was added to fix cells for 15 mins, then the mixture was removed and replaced with methanol for 15 min. Afterwards, remove methanol and stain 96 well plate with 0.5% crystal violet (500 mg in 100 mL of millQ water: methanol 4:1) for 20 mins. Remove the crystal violet solution and carefully rinse with an indirect flow of tap water, Invert plate and leave to dry on the bench, at room temperature. 200 μL of methanol was added to each well, and incubate the plate

with its lid on for 20 min at room temperature on a bench rocker with a frequency of 20 oscillations per minute. Put 96 well plate in a plate reader and scan at 570 nm wavelength.

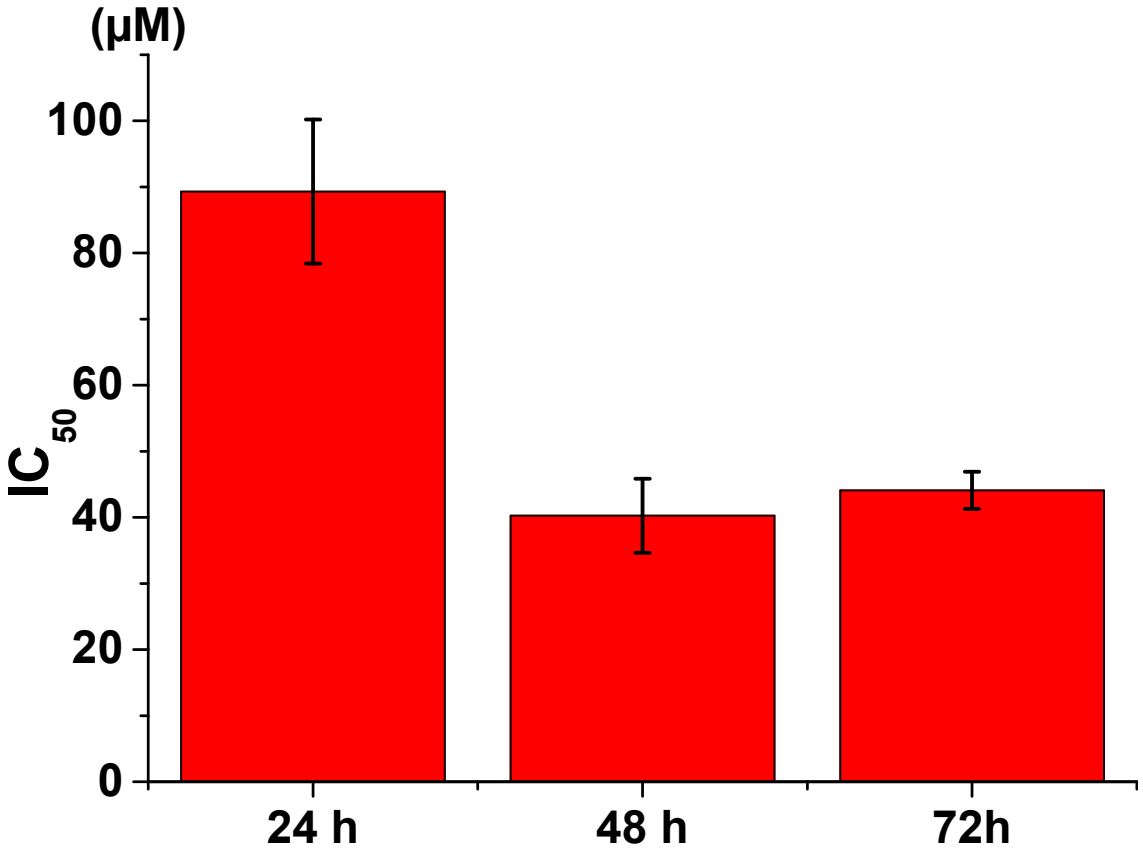

Figure S31. Crystal violet assay of EMT6 cells incubated with **[TNPH]Cl** for 24 hours (IC_{50} = 89.36 ± 10.91 μM), 48 hours (IC_{50} = 40.26 ± 5.60 μM), and 72 hours (IC_{50} = 44.10 ± 5.60 μM), the results are reported as a mean ± standard error. Error bars represents standard error of the mean.

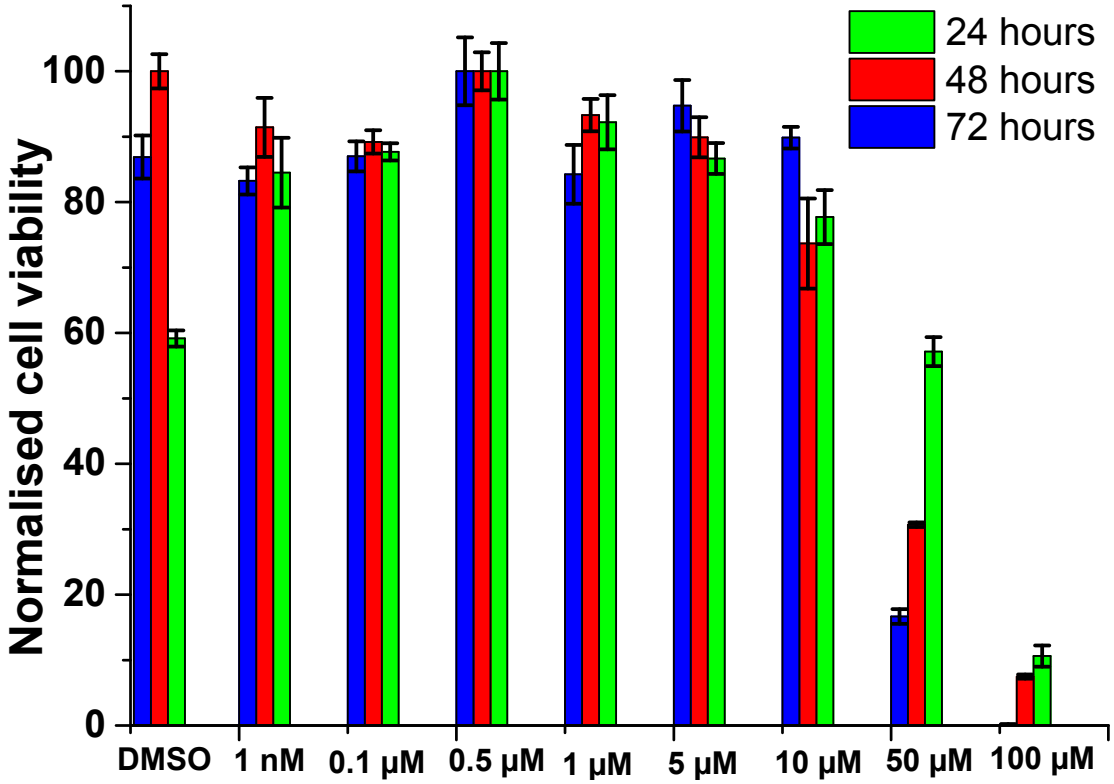

Figure S32. Cell viability of EMT6 cells incubated with **[TNPH]**Cl for 24 hours, 48 hours, and 72 hours, the results are reported as a mean ± standard error. Error bars represents standard error of the mean. N=3

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