Supporting Information

FETPY: a Diiron(I) Thio-Carbyne Complex with Prominent Anticancer Activity in Vitro and in Vivo

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Table of Contents

IR, NMR and MS Spectra (**Figures S1-S13**) S2−S8

X-Ray Crystallography (**Tables S1-S2**) S9

Behavior in Aqueous Media S10−S11

Biological Studies (**Figures S14-S19**, **Table S3**) S12−S18

Notes and References S19

IR, NMR and MS Spectra

Figure S1. Solid-state IR spectrum (650-4000 cm^{-1}) of $[Fe_2Cp_2(CO)_2(\mu\text{-}CO)(\mu\text{-}CSMe)]CF_3SO_3$, [**1**]$CF_3SO_3$.

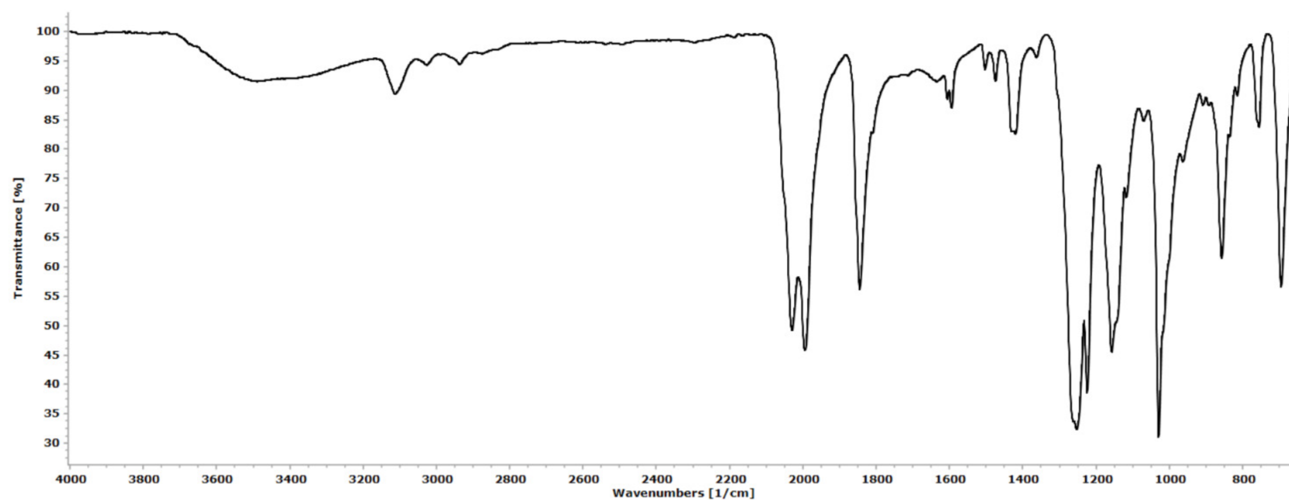


Figure S2. ^{1}H NMR spectrum (401 MHz, acetone-d$_6$) of [**1**]CF_3SO_3.

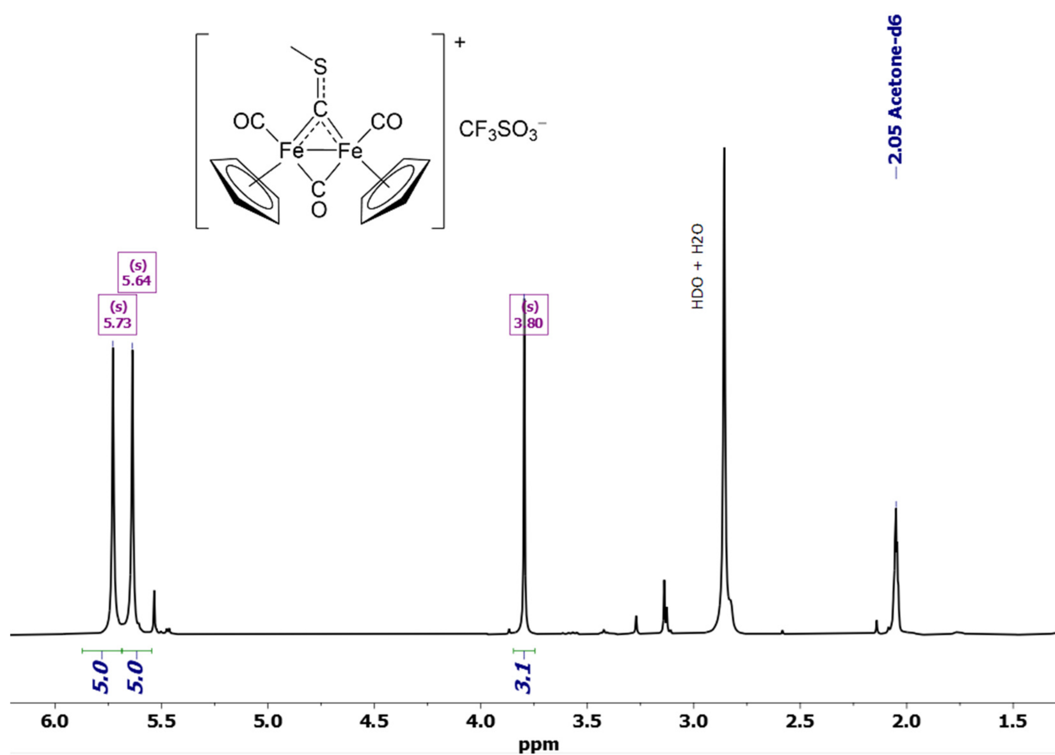


Figure S3. $^{13}C\{^{1}H\}$ NMR spectrum (101 MHz, acetone-d_6) of [**1**]CF_3SO_3.

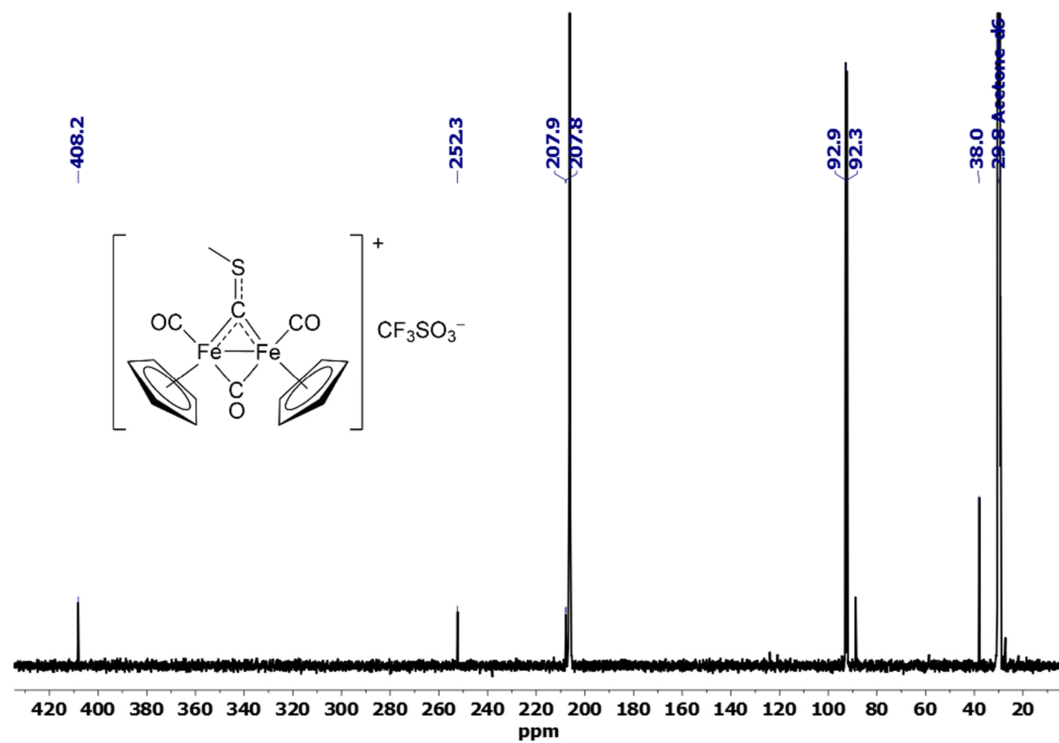


Figure S4. Solid-state IR spectrum (650-4000 cm^{-1}) of $[Fe_2Cp_2(CO)(DMAP)(\mu\text{-}CO)(\mu\text{-}CSMe)]CF_3SO_3$, [**2**]$CF_3SO_3$.

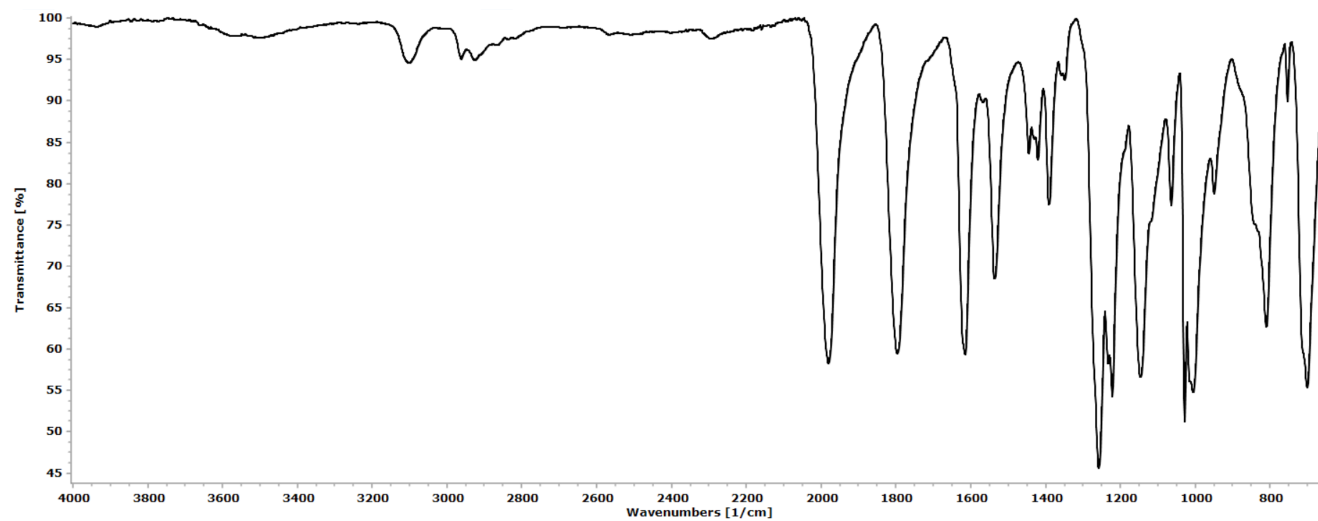


Figure S5. ^1H NMR spectrum (401 MHz, acetone-d_6) of [**2**]CF_3SO_3. Inset shows the S-Me resonance.

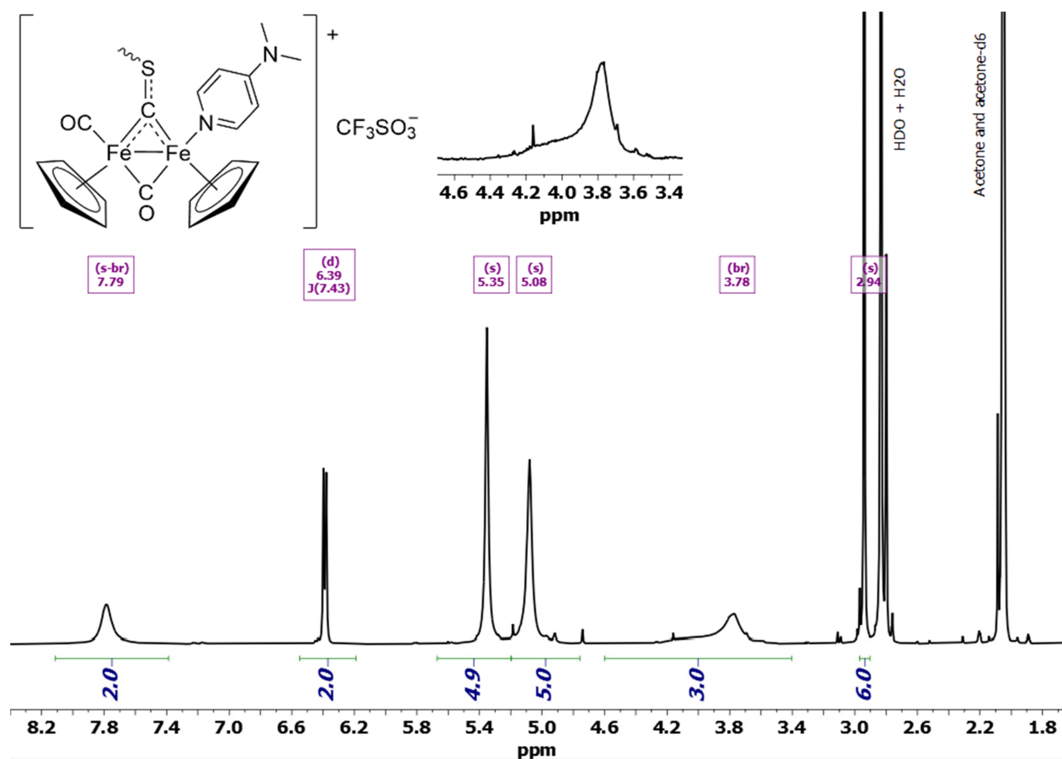


Figure S6. $^{13}C\{^1H\}$ NMR spectrum (101 MHz, acetone-d_6) of [**2**]CF_3SO_3.

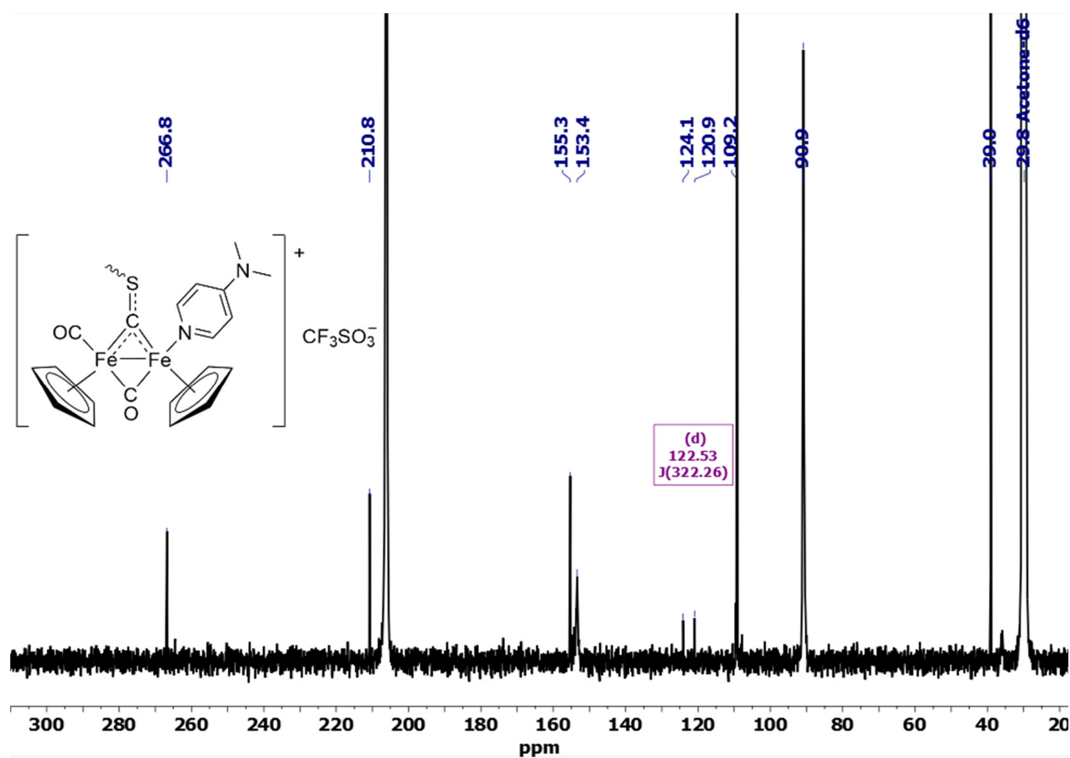


Figure S7. FIA-ESI(+)-MS spectrum of [**2**]CF_3SO_3 in MeOH in the 100-800 m/z range.

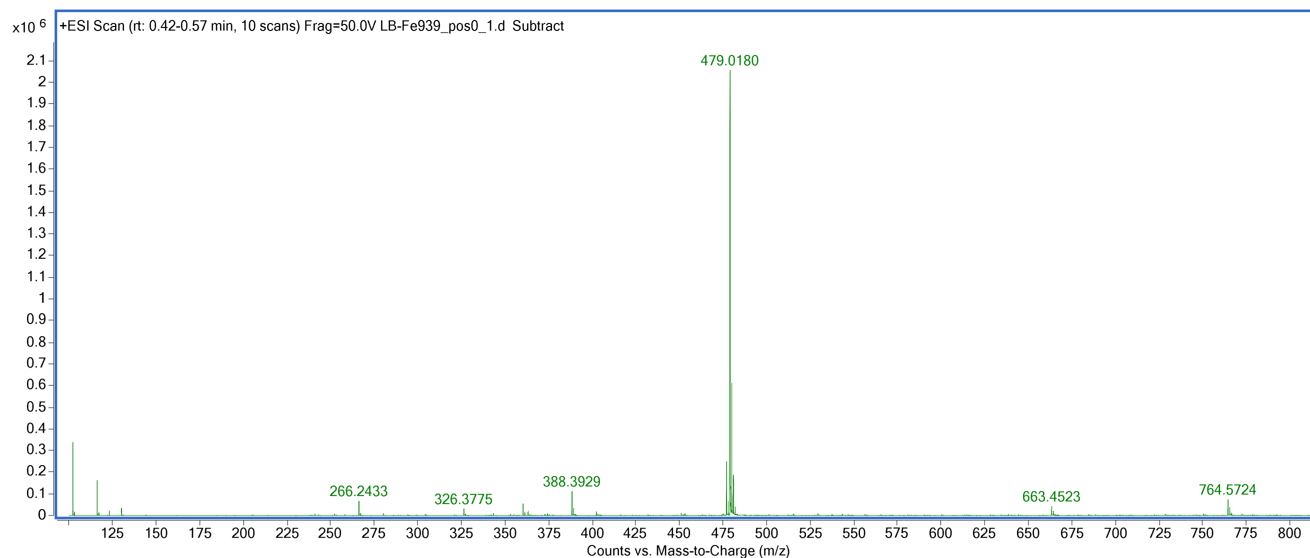


Figure S8. FIA-ESI(+)-MS spectrum of [**2**]CF_3SO_3 in MeOH (black line) and calculated isotopic pattern (red boxes). Calcd. base peak for [**2**]$^+$ ($C_{21}H_{23}Fe_2N_2O_2S$): 479.0178 Da. Score (exact mass + isotopic abundance): 99%. Difference (ppm): 1.09. Difference on the main peak (ppm): 1.5.

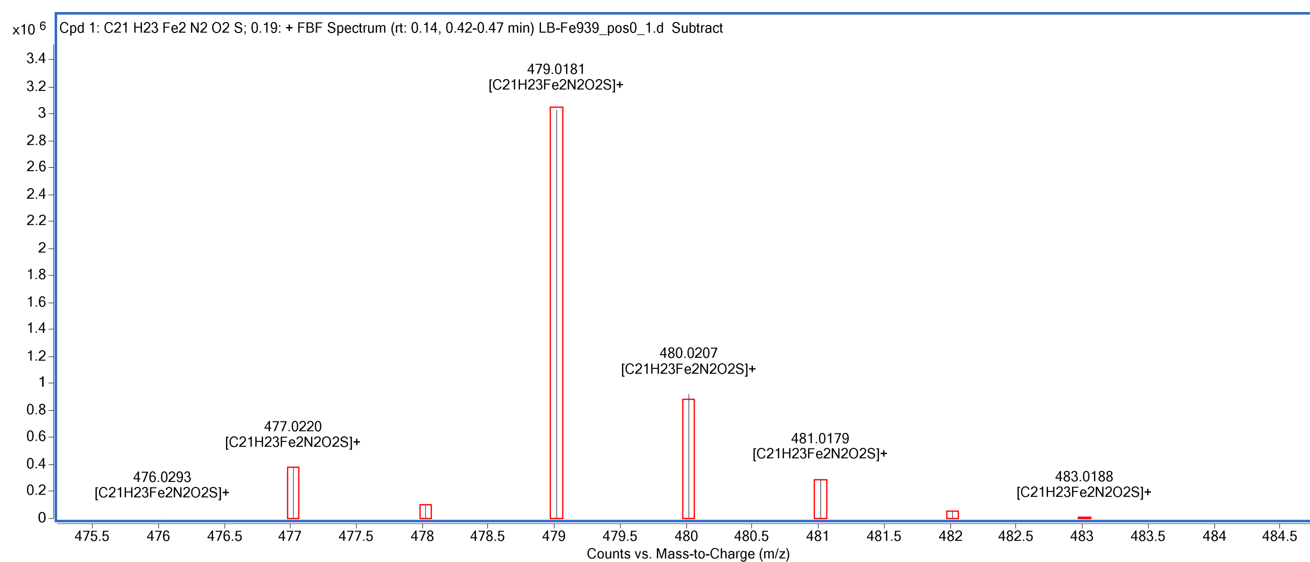


Figure S9. Solid-state IR spectrum (650-4000 cm^{-1}) of $[Fe_2Cp_2(CO)(\kappa P\text{-PTA})(\mu\text{-CO})(\mu\text{-CSMe})]CF_3SO_3$, [**3**]$CF_3SO_3$.

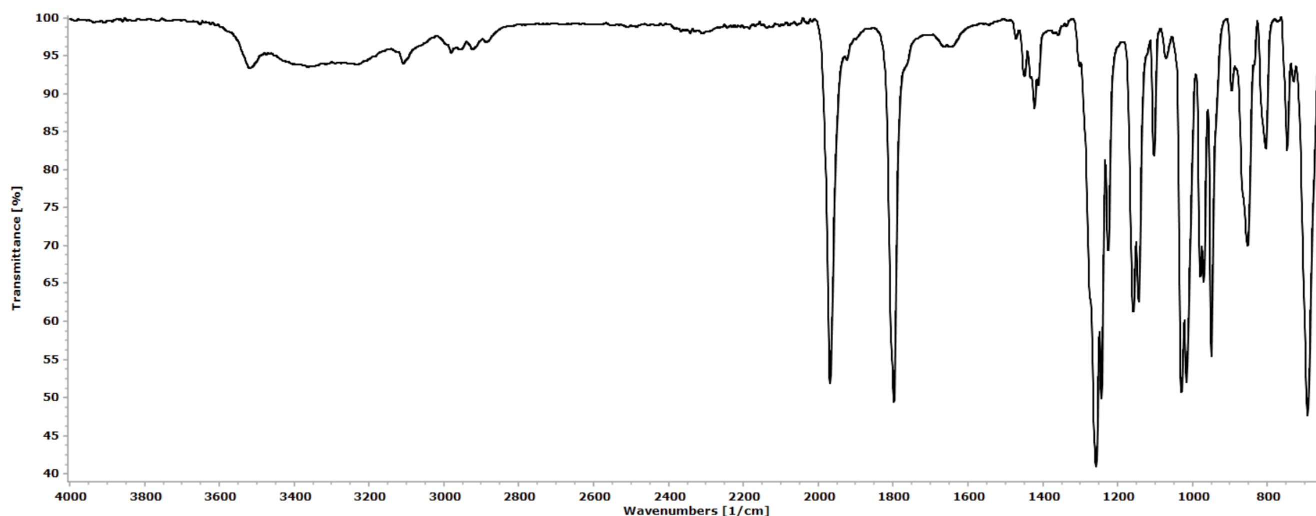


Figure S10. ^1H NMR spectrum (401 MHz, CD_3OD) of [**3**]CF_3SO_3.

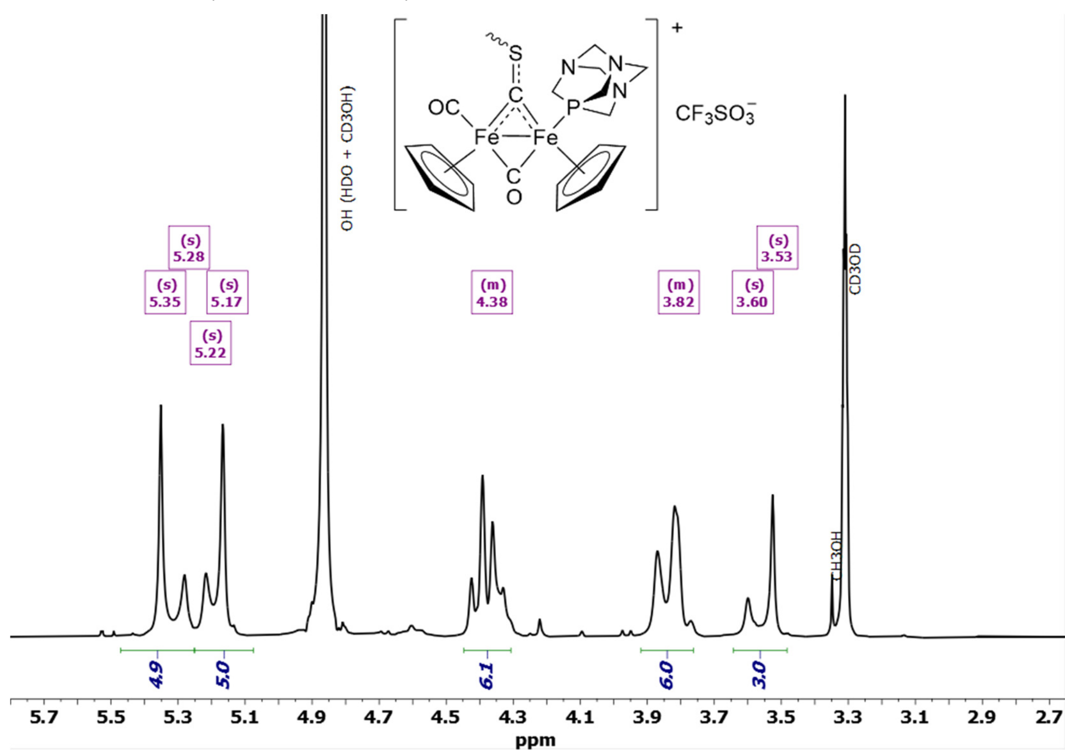


Figure S11. $^{13}C\{^{1}H\}$ NMR spectrum (101 MHz, DMSO-d$_6$) of [**3**]CF_3SO_3.

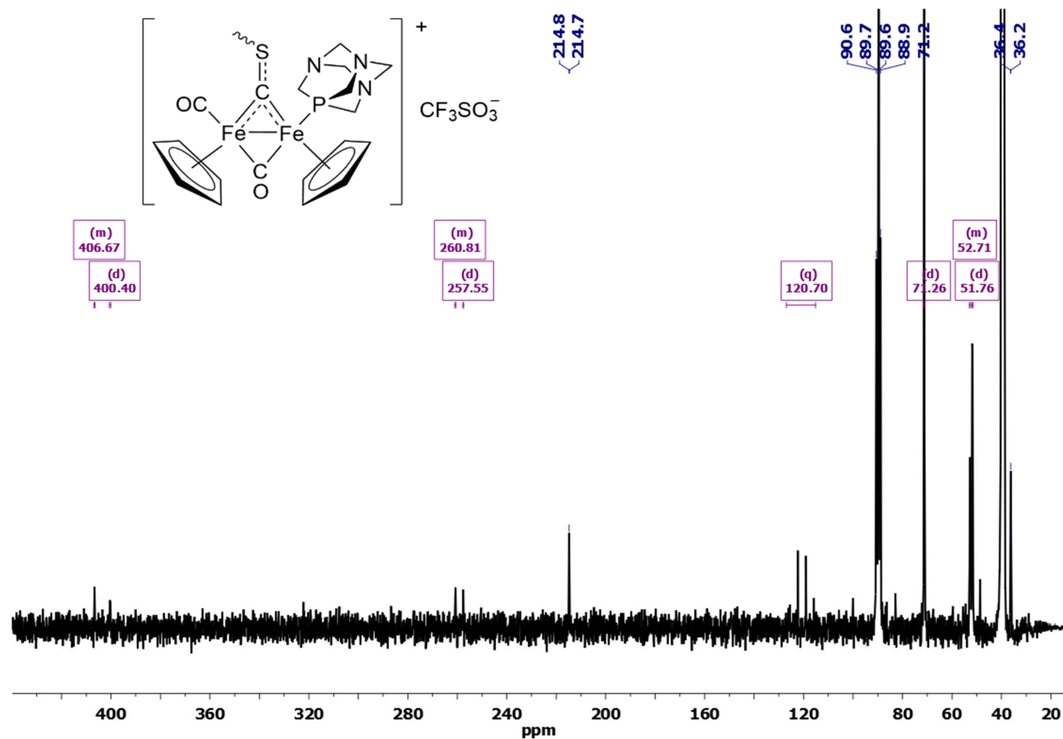


Figure S12. $^{31}P\{^{1}H\}$ NMR spectrum (162 MHz, CD_3OD) of [**3**]CF_3SO_3.

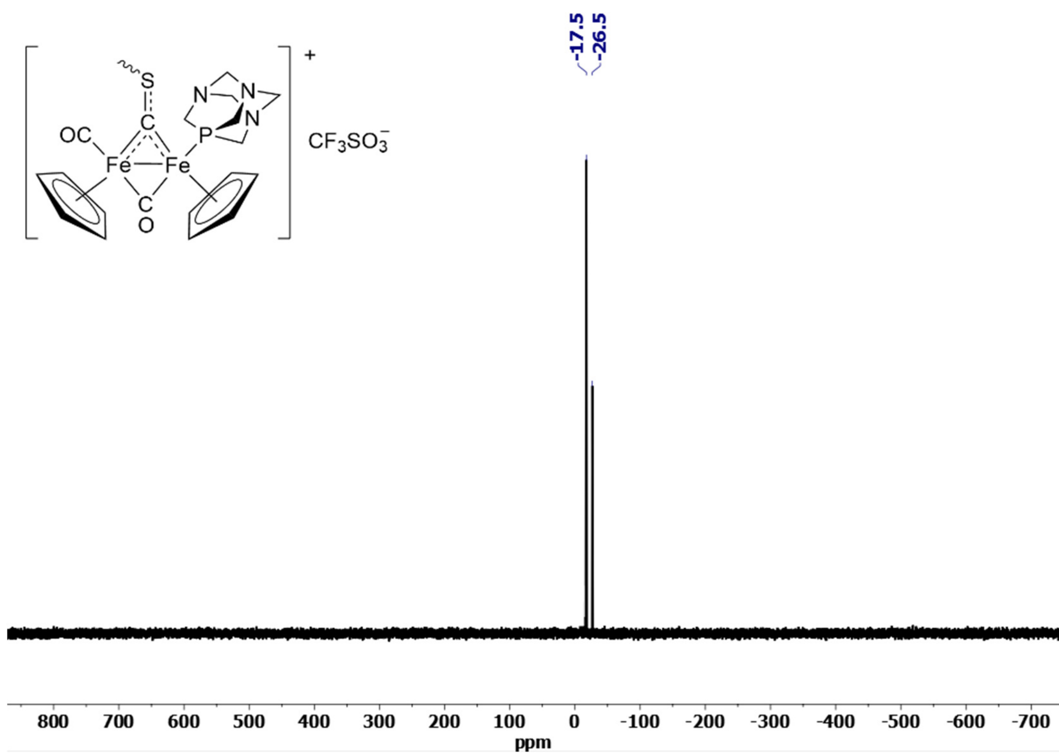
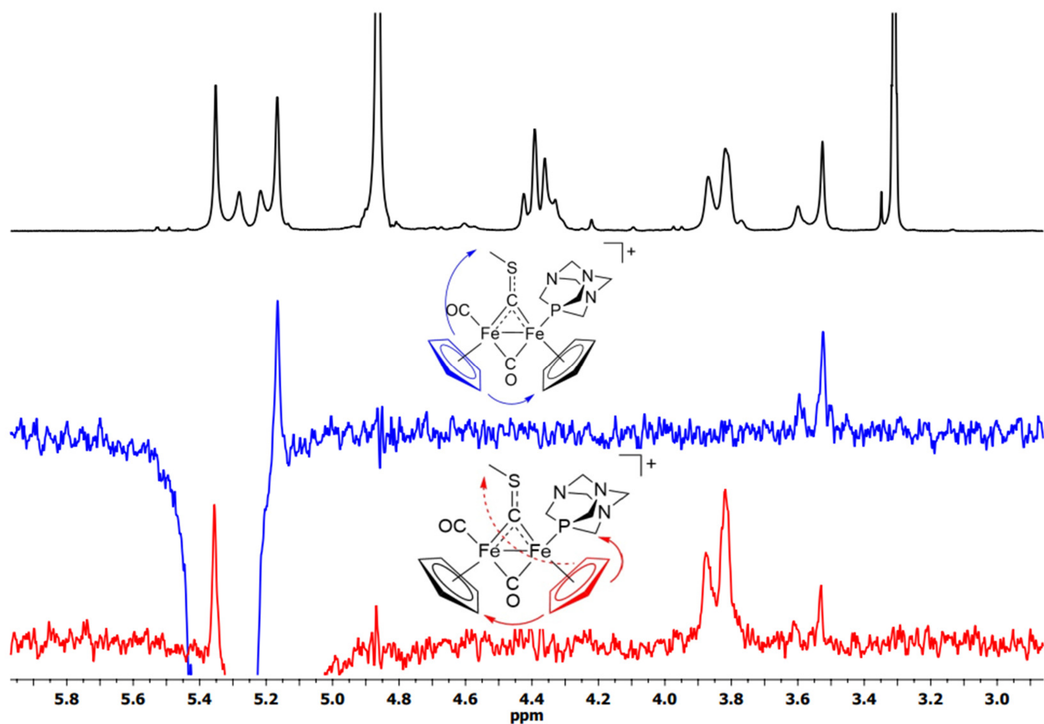


Figure S13. Black line: ^{1}H NMR spectrum (401 MHz, CD_3OD) of [**3**]CF_3SO_3. Blue line: ^{1}H NOESY with irradiation at 5.35 ppm (Cp of the *cis-E* isomer). Red line: ^{1}H NOESY with irradiation at 5.17 ppm (Cp^P of the *cis-E* isomer). Observed NOEs are indicated by the arrows (dotted line for the weaker NOE between Cp^P and SMe).



X-Ray Crystallography

Table S1. Hydrogen bonds for [**3**]$CF_3SO_3\cdot(H_2O)_{2.33}$ [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O(201)-H(201)...N(4)	0.84(2)	2.14(5)	2.944(15)	160(14)
O(202)-H(202)...O(203)	0.84(2)	1.93(5)	2.714(12)	154(11)
O(202)-H(212)...N(2)	0.84(2)	2.04(5)	2.855(11)	161(14)
O(203)-H(203)...O(203)#2	0.84(2)	2.306(17)	2.75(2)	114.1(13)
O(203)-H(213)...O(101)	0.84(2)	2.12(4)	2.936(11)	163(11)
O(204)-H(204)...O(103)	0.84(2)	2.02(3)	2.833(12)	166(12)
O(204)-H(214)...O(202)	0.83(2)	1.93(3)	2.760(12)	171(13)

Symmetry transformations used to generate equivalent atoms: #1 -x+1,-y+1,-z+1

Table S2. Crystal data and measurement details for [**3**]$CF_3SO_3\cdot(H_2O)_{2.33}$.

Formula	$C_{21}H_{29.67}F_3Fe_2N_3O_{7.33}P_2S$
FW	705.27
T, K	100(2)
λ, Å	0.71073
Crystal system	Orthorhombic
Space group	*Pnma*
a, Å	19.0064(19)
b, Å	27.332(3)
c, Å	15.1618(15)
Cell Volume, Å^3	7876.3(13)
Z	12
D_c, g·cm^{-3}	1.784
μ, mm^{-1}	1.396
F(000)	4336
Crystal size, mm	0.18×0.16×0.13
θ limits,°	1.490–25.097
Reflections collected	86123
Independent reflections	7137 [R_{int} = 0.0828]
Data / restraints /parameters	7137 / 631 / 645
Goodness on fit on F^2	1.325
R_1 ($I > 2\sigma(I)$)	2347
wR_2 (all data)	0.0532
Largest diff. peak and hole, e Å^{-3}	1.425 / –1.658

Behavior in Aqueous Media

Solubility in water (D_2O). The selected compound was suspended in a D_2O solution (0.7 mL) containing dimethyl sulfone (Me_2SO_2; $4.0 \cdot 10^{-3}$ mol·L^{-1}) and stirred at room temperature (*ca.* 21 °C) for 3 h. The saturated solution was filtered over celite and analyzed by ^{1}H NMR spectroscopy (delay time = 3 s, number of scans = 20). The concentration (= solubility) was calculated by the relative integral with respect to Me_2SO_2 as internal standard [δ/ppm = 3.14 (s, 6H)] (Table 1). NMR data are given below.

Octanol-water partition coefficient (Log P_{ow}). Partition coefficients (P_{ow}), defined as $P_{ow} = c_{org}/c_{aq}$, where c_{org} and c_{aq} are the molar concentrations of the selected compound in the n-octanol and aqueous phase, respectively, were determined by the shake-flask method and UV-Vis measurements, according to a previously described procedure.**Errore. Il segnalibro non è definito.**[1] All operations were carried out at room temperature (*ca*. 21 °C). Stock solutions of the diiron compounds were prepared in octanol-saturated. The wavelength corresponding to a well-defined maximum of shoulder absorption of each compound (340–350 nm range) was used for UV-Vis quantitation. The procedure was performed in triplicate for each sample (from the same stock solution); results are given as mean ± standard deviation (Table 1 in the main text).

Stability assessment in D_2O/CD_3OD at 37 °C. Compounds [**1,3**]CF_3SO_3 were dissolved in a D_2O solution containing Me_2SO_2 ($4.0 \cdot 10^{-3}$ mol·L^{-1}, 0.75 mL) while [**2**]CF_3SO_3 was dissolved in CD_3OD [2] then diluted with the D_2O/Me_2SO_2 solution (3/2 V/V ratio). The solutions ($c_{Fe2} \approx 5 \cdot 10^{-3}$ M, 0.75 mL) were filtered over celite and analyzed by ^{1}H and ^{31}P{^{1}H} NMR (delay time = 3 s; number of scans = 20). Next, the solutions were heated at 37 °C for 48 h. After cooling to room temperature, the final solutions were separated from a minor amount of brown precipitate by filtration over celite and the NMR analysis was repeated. In each case, no new {FeCp} species was identified in solution. The residual amount of starting material was calculated with respect to Me_2SO_2 as internal standard (Table 1 in the main text). NMR data and other experimental details are given below. ^{1}H NMR chemical shifts in D_2O/CD_3OD mixtures are referenced to the Me_2SO_2 peak as in pure D_2O.

Stability assessment in cell culture medium at 37 °C. Deuterated cell culture medium (DMEM-d) was prepared by dissolving powdered DMEM cell culture medium (1000 mg/L glucose and L-glutamine, without sodium bicarbonate and phenol red; D2902 - Sigma Aldrich) in D_2O (10 mg/mL). The solution was treated with Me_2SO_2 ($6.6 \cdot 10^{-3}$ mol·L^{-1}) and KH_2PO_4 / Na_2HPO_4 as pH buffer (0.10 M total phosphate, pD = 7.5 [3]), then stored at 4 °C under N_2. Solutions of diiron compounds in DMEM-d ([**1**]$^+$, [**3**]$^+$) or DMEM-d/CD_3OD 2:1 *V/V* ([**2**]$^+$) were prepared and treated as previously described. The residual amount of starting material in solution after 24 h at 37 °C was calculated with respect to Me_2SO_2 as internal standard (Table 1 in the main text).

Carbon monoxide release. In a 15x45 mm screw neck glass vial (5.0 mL total volume), the selected compound was accurately weighted (*ca.* 4 mg), dissolved in water or in the appropriate MeOH/water mixture as in the related NMR experiments (4.0 mL total liquid volume; $c_{Fe2} \approx 1.2 \cdot 10^{-3}$ mol·L^{-1}). Next, the vial was sealed with a PTFE/silicone septum screw cap and maintained at 37 °C for 24 h, by full immersion into a thermostated water bath. After cooling to room temperature, the headspace was sampled with a gas-tight microsyringe (250 μL) and analysed by GC-TCD. Measurements were performed in duplicate for each compound. The amount of carbon monoxide (n_{CO}, mmol) was calculated based on a calibration curve obtained from analyses of known CO/air mixtures (0.1-1.0 mmol·L^{-1}), assuming ideal gas behavior. The number of equivalents of carbon monoxide released ($eq_{CO} = n_{CO}/n_{Fe2}$) was calculated with respect to the initial amount of the complex (Table 1).

NMR data and other experimental details.

[**1**]CF_3SO_3. Red-orange solution. ^{1}H NMR (D_2O): δ/ppm = 5.48, 5.43 (s-br, 10H); 3.62 (s, 3H).

[**2**]CF_3SO_3. Brown solution. ^{1}H NMR (D_2O/CD_3OD 3:2 *V*/*V*): δ/ppm = 7.75 (br, 2H), 6.33 (d, *J* = 6.2 Hz, 2H), 5.23 (s, 5H), 4.98*, 3.9–3.6 (br, 3H), 2.90 (s, 6H). *Over HDO peak. Other products identified (48 h): DMAP.

DMAP. ^{1}H NMR (D_2O/CD_3OD 3:2 *V*/*V*): δ/ppm = 8.09 (d, *J* = 7.3 Hz), 6.91 (d, *J* = 7.2 Hz), 3.21 (s).

[**3**]CF_3SO_3. Green-brown solution. ^{1}H NMR (D_2O): δ/ppm = 5.35, *5.29* (s-br, 5H); *5.18*, 5.14 (s-br, 5H); 4.42–4.28 (m, 6H); 3.85–3.72 (m, 6H); *3.55*, 3.49 (s, 3H); *cis*-*E*/*cis*-*Z* isomer ratio = 3 (0-48 h). ^{31}P{^{1}H} NMR (D_2O): δ/ppm = − 12.5, − *21.0*. Other products identified (48 h): 1,3,5-triaza-7-phosphaadamantane-7-oxide (O=PTA).

O=PTA. ^{1}H NMR (D_2O): δ/ppm = 3.94 (d, *J* = 10.9 Hz). ^{31}P{^{1}H} NMR (D_2O): δ/ppm = − 2.9. ^{1}H NMR (D_2O/CD_3OD 6:1 *V*/*V*): δ/ppm = 4.03 (d, *J* = 10.4 Hz). ^{31}P{^{1}H} NMR (D_2O/CD_3OD 6:1 *V*/*V*): δ/ppm = 1.9.

Biological Studies

Figure S14. The effect of [**1**]CF_3SO_3 (black dots), [**2**]CF_3SO_3 (white squares) and [**3**]CF_3SO_3 (white triangles) on the viability of human cancer cell lines (HCT 116, MCF7 and A2780) and human fetal lung fibroblasts (MRC5) was assessed by two different viability assays after 72 hours of treatment. Left side represents results of MTT assay, while the right side represents results of CV assay. The data is expressed as percentage of viability of untreated cells that represent control (100%). One representative out of three independent experiments is shown as mean ± SD of triplicate cultures (* $p < 0.05$ in comparison to control).

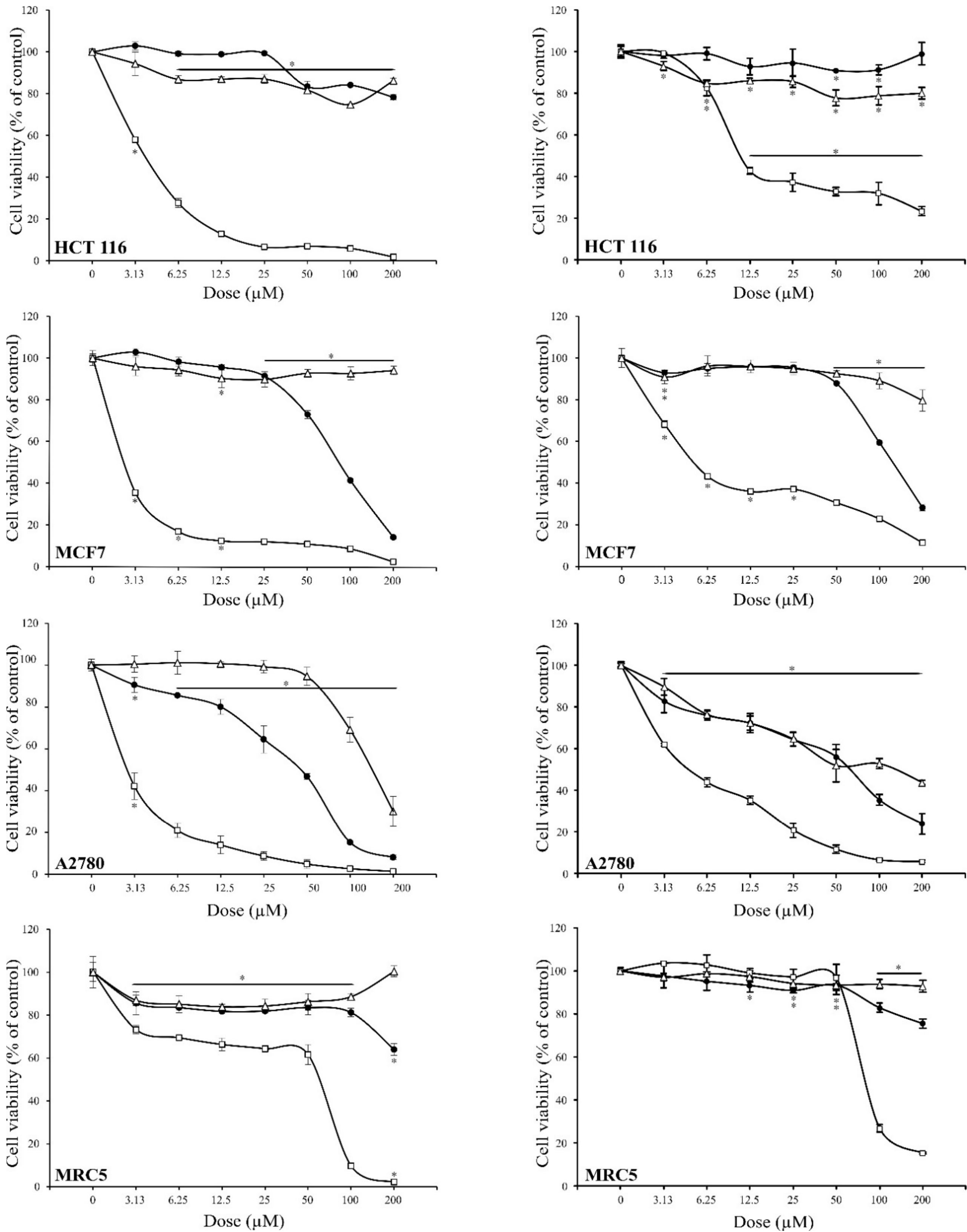


Figure S15. A2780 cells were treated with IC_{50} value of [**2**]CF_3SO_3 for 48 hours and stained with (A) Annexin V (AnnV)/propidium iodide (PI); (B) Apostat; (C) PI and (D) Acridine orange (AO). (E) A2780 cells were treated with IC_{50} value of [**2**]CF_3SO_3 for 72 hours and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). All samples were analyzed by flow cytometry, except C, where cells were analyzed by fluorescent microscopy.

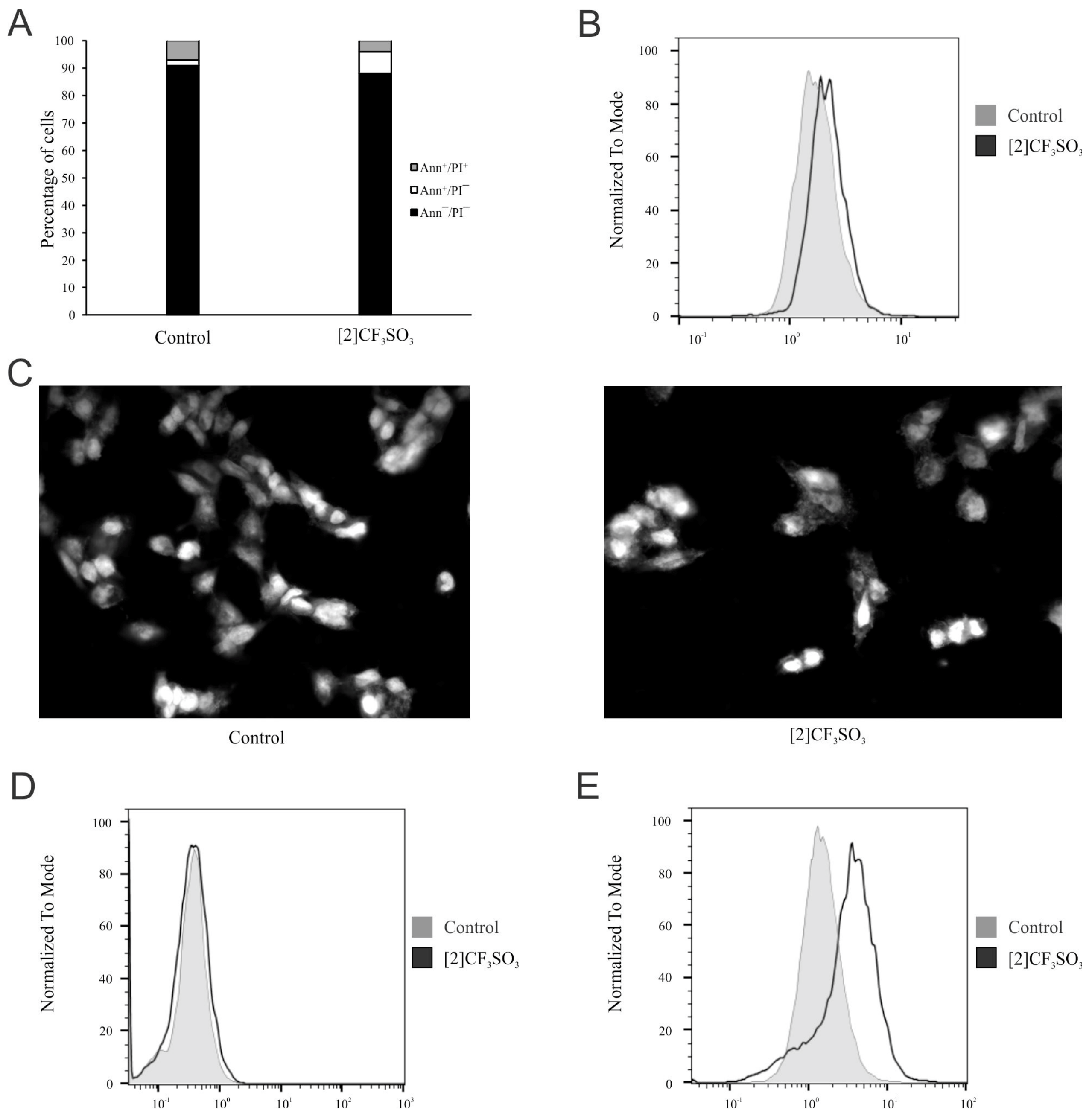


Figure S16. The effect of [**2**]CF_3SO_3 on the viability of mouse cancer cell lines (B16-F1 black dots, B16-F10 white squares and 4T1 white triangles) and mouse embryonic fibroblasts (NIH 3T3 black rhombuses) was assessed by two different viability assays after 72 hours of treatment. Left side represents results of MTT assay, while the right side represents results of CV assay. The data is expressed as percentage of viability of untreated cells that represent control (100%). One representative out of three independent experiments is shown as mean ± SD of triplicate cultures (* $p < 0.05$ in comparison to control).

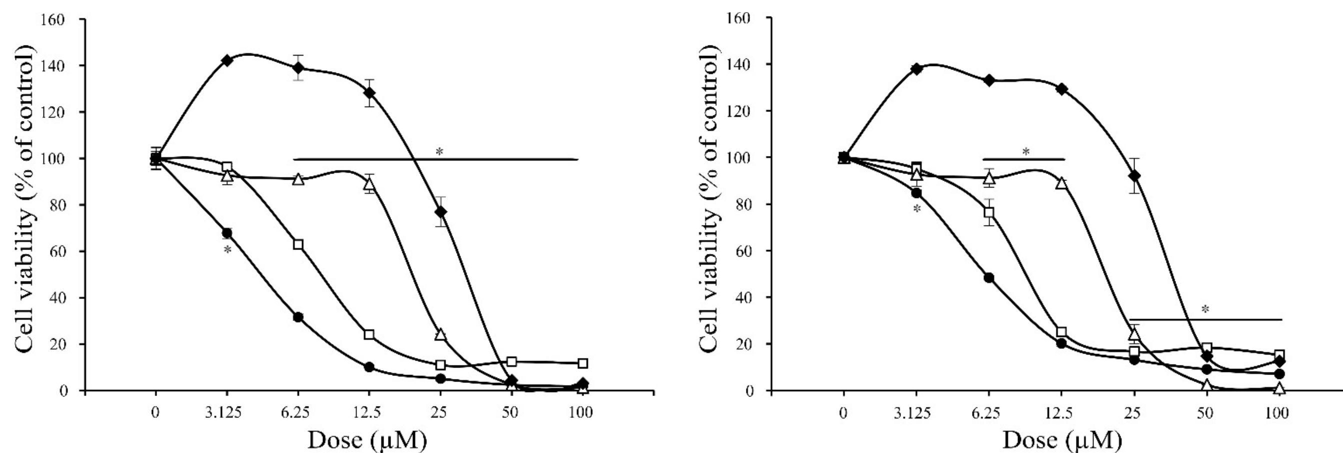


Table S3. IC_{50} values of [**2**]CF_3SO_3 on mouse cell lines after 72 hours of treatment ± SD (μM)

Cell line	Viability assay	IC_{50} ± SD
B16-F1 Low-invasive melanoma	MTT	4.5 ± 0.4
	CV	5.4 ± 0.5
B16-F10 High-invasive melanoma	MTT	5.9 ± 0.4
	CV	9.3 ± 0.4
4T1 Breast cancer	MTT	27.5 ± 1.5
	CV	34.8 ± 0.1
NIH 3T3 Embryonic fibroblasts	MTT	36.1 ± 0.5
	CV	39.7 ± 0.3

Figure S17. B16-F1 cells (represented on the left side) and B16-F10 cells (represented on the right side) were treated with IC_{50} value of [**2**]CF_3SO_3 for 72 hours and stained with (A) Annexin V (AnnV)/propidium iodide (PI); (B) Apostat; (C) PI and (D) Acridine orange (AO). All samples were analyzed by flow cytometry, except C, where cells were analyzed by fluorescent microscopy. E) B16-F1 cells were treated with IC_{50} value of [**2**]CF_3SO_3 in the presence of autophagy inhibitor, chloroquine. After 72 hours of treatment, cell viability was assessed using CV assay. The data is expressed as percentage of viability of untreated cells that represent control (100%). One representative out of three independent experiments is shown as mean ± SD of triplicate cultures (*** $p < 0.001$ in comparison to treatment with IC_{50} value of [**2**]CF_3SO_3 without chloroquine).

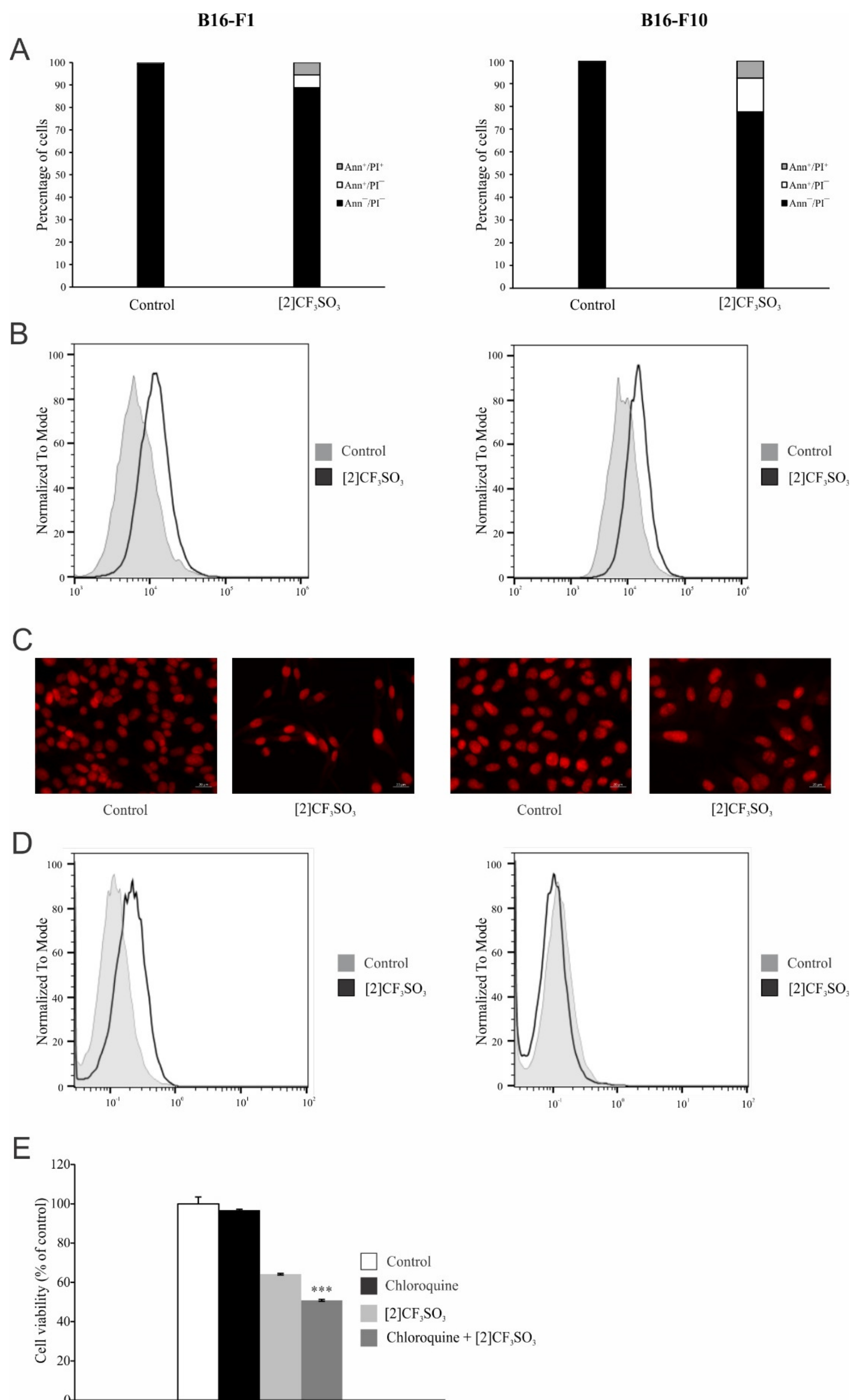


Figure S18. B16-F1 cells (represented on the left side) and B16-F10 cells (represented on the right side) were treated with IC_{50} value of [**2**]CF_3SO_3 for 72 hours. Cells were tested for melanin content (A) and tyrosinase activity (B). The data is expressed as percentage of melanin content/tyrosinase activity of untreated cells that represent control (100%). One representative out of three independent experiments is shown as mean ± SD of triplicate cultures.

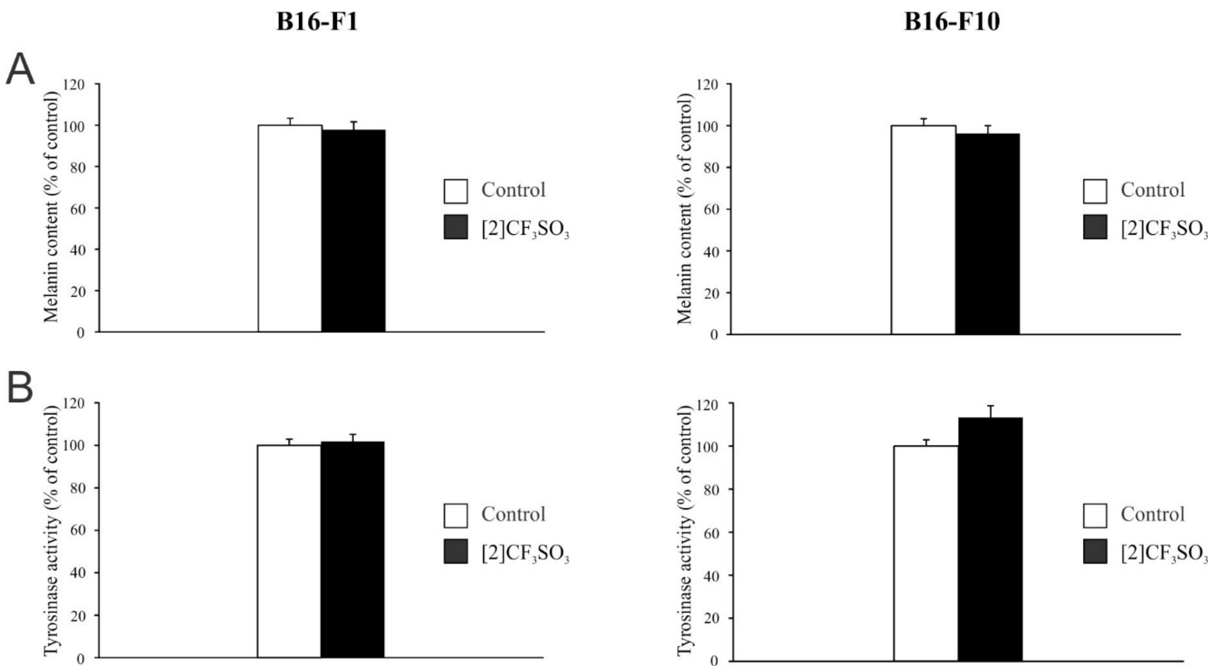
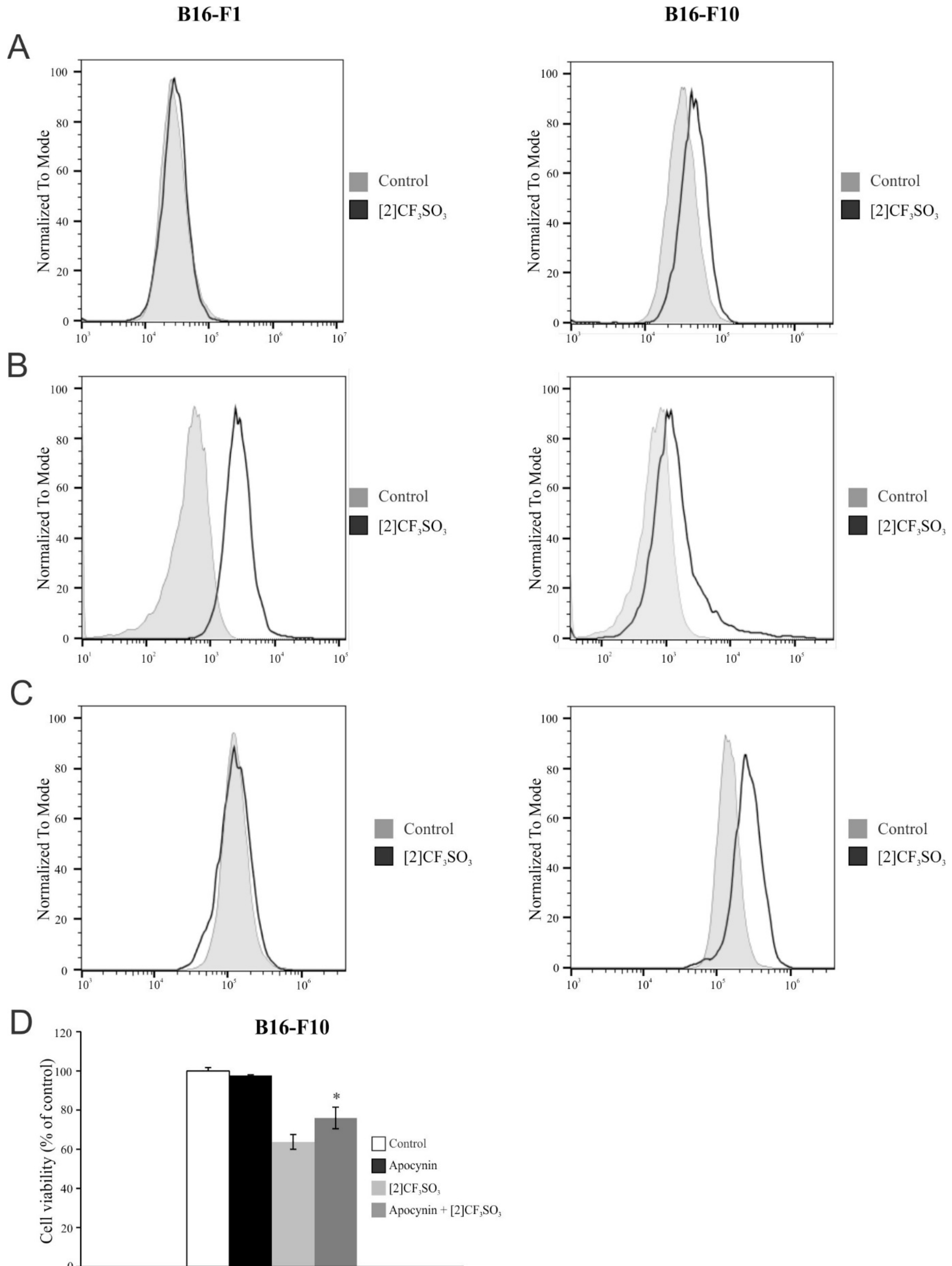


Figure S19. B16-F1 cells (represented on the left side) and B16-F10 cells (represented on the right side) were treated with IC_{50} value of [**2**]CF_3SO_3 for 72 hours, stained with (A) Dihydrorhodamine 123 (DHR); (B) Diaminofluorescein (DAF) FM diacetate and (C) dihydroethidium (DHE) and then analyzed by flow cytometry. (D) B16-F10 cells were treated with IC_{50} value of [**2**]CF_3SO_3 in the presence of NADPH oxidase 2 (NOX2) inhibitor, apocynin. After 72 hours of treatment, cell viability was assessed using CV assay. The data is expressed as percentage of viability of untreated cells that represent control (100%). One representative out of three independent experiments is shown as mean ± SD of triplicate cultures (* $p < 0.05$ in comparison to treatment with IC_{50} value of [**2**]CF_3SO_3 without apocynin).



Notes and references

1 (a) OECD Guidelines for Testing of Chemicals. In OECD, Paris: **1995**; Vol. 107; (b) Dearden, J. C.; Bresnen, G. M. *Quant. Struct.-Act. Relat.* **1988**, *7*, 133.

2 Methanol was used as a co-solvent to prepare solutions suitable for ^{1}H NMR analysis (> 3 mM); the methanol/water ratio was selected with respect to the water solubility of the compound.

3 Calculated by the formula pD = pH* + 0.4, where pH* is the value measured for H_2O-calibrated pH-meter. Covington, A. K.; Paabo, M.: Robinson, R. A.; Bates, R. G. *Anal. Chem*. **1968**, *40*, 700.