Supplementary Information

Fluorogenic Chemical Tools to Shed Light on CES1-Mediated Adverse Drug Interactions

Carolyn J. Karns,[a] Taylor P. Spidle,[a,b,+] Emmanuel Adusah,[b,+] Mingze Gao,[a] Jennifer E. Nehls,[a] and Michael W. Beck[b,*]

[a.]Department of Chemistry and Biochemistry, Eastern Illinois University, Charleston, IL 61920 (USA)
[b.]Department of Biological Sciences, Eastern Illinois University, Charleston, IL 61920 (USA)
[+]These authors contributed equally to this work.
*Email: MBeck2@EIU.edu

General materials and methods. Unless otherwise noted, all purchased reagents were used as received without further purification. Millipore filtered water was used as the water source for all experiments. ^{1}H NMR spectra were recorded on a Bruker Advance 400 MHz spectrometer. ^{1}H and ^{13}C NMR chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) with shifts corrected using residual solvent peaks. In reported spectral data, the format (δ) chemical shift (multiplicity, J values in Hz, integration) is used with the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectrometry (MS) studies were carried out by personnel at the University of South Carolina Mass Spectrometry Center under a contract research agreement. The accurate mass data was acquired on a VG-70S magnetic sector mass spectrometer (Waters) by direct probe introduction and electron ionization (EI) at 70 eV ionization energy. All *in vitro* fluorescence experiments were carried out using an Agilent BioTek Synergy H1 Hybrid Multi-Mode Reader equipped with Variable Bandwidth Monochromators and Red Extended PMT (Santa Clara, CA). When noted the plate reader was equipped with a blue/green filter cube (Agilent, 8040501) and the green filter set (λ_{Ex} = 485/20, λ_{Em} = 528/20, dichroic mirror 510 nm) was used for improved sensitivity. All fluorescence experiments utilized 96 well Greiner Bio-One black μclear bottom microplates (Monroe, NC) and top read at a height of 6.25 mm. Fluorescence microscopy images were obtained on a Leica inverted fluorescence (epifluorescence) DM IL LED microscope equipped with an 60x oil objective. Green channel images were obtained using a Semrock YFP-2427B Brightline Long Pass Filter Set (λ_{ex} = 480 nm to 510 nm; dichroic mirror = 510 nm; $\lambda_{em} \geq$ 560 nm). All images were taken using identical conditions (exposure time, light source intensity, digital gain, and magnification) in relation to the relevant control conditions. Images were analyzed using ImageJ.[1] Cell counts were performed manually using a hemocytometer. All error bars are +/- standard deviation. IUPAC names for compounds were generated using Marvin JS (Version 24.1.0, ChemAxon, Budapest, Hungary).

Molecular Docking Studies

Rigid receptor-flexible ligand docking was carried out in AutoDock Vina[2,3] version 1.2.3 using the Webina interface[4] with an exhaustiveness of 64. The receptor was prepared from Chain A of human CES1 (PDB ID 1DR0)[5] using PyMOL (Schrodinger, LLC. 2010. The PyMOL Molecular Graphics System, Version 3.0.0) and protonated at pH 7.4 when it was converted to pdbqt file in Webina. Receptors (MCP-Me and MCP-Et) were geometry optimized using the MMFF94s forcefield in Avogadro[6] and converted to pdbqt files in Webina. The entire active site was placed in the docking box (grid center: X=1, Y=43,

Z=43, grid size: X=30, Y=20, Z=20, grid space: 0.375). PyMOL was used for the analysis of results and generation of figure images.

Synthesis of MCP-Me (3'-methoxy-3-oxo-3H-spiro[2-benzofuran-1,9'-xanthen]-6'-yl methyl carbonate). MCP-Me was prepared from 3-O-Methylfluoroscein (MOF)[7] by adapting previously reported procedures.[8] To summarize, in an oven dried round bottom flask equipped with a magnetic stir bar, MOF (0.3427 g, 0.99 mmols) was added and the flask was sealed with a rubber septa. The reaction was placed under a nitrogen atmosphere, 15 mL of anhydrous THF was added, and the reaction was cooled on ice. Triethylamine (0.28 mL, 2.0 mmol) was added followed by dropwise addition of methyl chloroformate (0.23 mL, 3.0 mmol). The ice bath was allowed to melt as the reaction continued. After 12 h, the solvent was removed under vacuum and the crude product was purified via silica column flash chromatography (20% EtOAc in Hexanes) and recrystallized twice from DCM and hexanes yielding 0.1015 g of product (25.4% yield). ^{1}H NMR (400 MHz, CD_2Cl_2) δ (ppm): 8.02 (d, J=8.0 Hz, 1H), 7.67 (m, 2H), 7.18 (m, 2H), 6.90 (m, 1H) 6.83 (m, 1H), 6.74 (m, 1 H), 6.67 (m, 1H) 3.90 (s, 3H), 3.84 (s, 3H) ^{13}C NMR (400 MHz, CD_2Cl_2) δ (ppm): 169.50, 162.20, 154.17,153.38, 152.89, 152.81, 135.77, 130.51, 129.66, 129.53, 127.06, 125.53, 124.47, 117.73, 117.44, 112.47, 111.51, 110.32, 101.42, 82.67, 56.21, 56.16. HRA-MS(+) calculated for formula $C_{23}H_{16}O_7$ 404.0896; found 404.0900.

Synthesis of MCP-Et (3'-methoxy-3-oxo-3H-spiro[2-benzofuran-1,9'-xanthen]-6'-yl ethyl carbonate). MCP-Et was synthesized from MOF following the same procedure of MCP-Me using 0.1515 g (0.43 mmol) of MOF, 0.12 mL (0.86 mmol) of triethylamine, 5 mL of anhydrous THF, and 0.12 mL (1.3 mmol) of ethyl chloroformate. The crude produce was purified via silica column flash chromatography (20% EtOAc in Hexanes) yielding 0.1386 g of product (76.5% yield). ^{1}H NMR (400 MHz, d6-DMSO) δ (ppm): 8.05 (d, J=8 Hz, 1H), 7.79 (m, 2 H), 7.37 (m, 2 H), 7.04 (m, 1 H), 6.98 (d, J=4 Hz, 1H), 6.88 (d, J=8 Hz, 1H), 6.74 (m, 2H), 4.30 (q, J=4 Hz, 2H), 3.38 (s, 3H), 1.30 (t, J=8 Hz, 3 H). ^{13}C NMR (400 MHz, d6-DMSO) δ (ppm): 168.50, 161.25, 152.43, 152.32, 151.99, 151.55, 151.06, 135.90, 130.44, 129.06, 125.66, 124.92, 124.07, 117.76, 116.76, 112.43, 110.52, 109.91, 100.85, 81.58, 65.00, 55.75, 13.96. HRA-MS(+) calculated for formula $C_{24}H_{18}O_7$ 418.1053; found 418.1048.

Fluorescence spectroscopy of MOF, MCP-Me, and MCP-Et. Fluorescence spectra were recorded using 200 µL of 1 µM MOF, MCP-Me or MCP-Et in 1X PBS (Fisher) in a 96 well plate and excited from 400 to 525 nm with the emission recorded at 550 nm. Emission spectra of MOF, MCP-Me, and MCP-Et from 450 to 700 nm were collected after excitation at 400 nm. Values are reported relative to the fluorescence of MOF.

Stability of MCP-Me and MCP-Et across pH. Stability at variable pH was determined using a modified previous reported procedure.[8] To summarize, 1 µM MOF, MCP-Me, or MCP-Et with 0.1% DMSO were prepared in 20mM glycine (pH 3.0), acetate (pH 4.0-5.0), phosphate (pH 6.0-8.0), Tris (pH 9.0), or CAPS (pH 10.0) buffer and incubated for 30 min at 37°C. After incubation, the fluorescence intensity (λ_{ex}=460/20 nm; λ_{ex}=550/50 nm) was recorded in triplicate.

Solution stability of MCP-Me and MCP-Et. Stability was determined using a modified previous reported procedure.[8] To summarize, 1 µM MOF, MCP-Me, or MCP-Et were incubated at 37°C in 1X PBS (Fisher) or at in Gibco Fluorobrite DMEM (Fisher) supplemented with 20 mM HEPES at pH 7.4 for 3 h with the fluorescence intensity (λ_{ex}=460/20 nm; λ_{ex}=550/50 nm) recorded every 1 min in triplicate.

Units of CES activity determination. The units per µL of human recombinant enzyme CES1 (453320, Corning, Corning, NY) and CES2 (453322, Corning) were determined using the manufacturer's protocol in 1X PBS, pH 7.4. Units of activity were utilized instead of mg of protein due to the known variability of *in vitro* CES activity.[8,10–12]

Hydrolysis of MCP-Me and MCP-Et by CESs. 100 µL of 1 µM MCP-Me or MCP-Et in 1X PBS were added to a 96 well plate before addition of 0.5 units of CES1, CES2, or no enzyme in 100 µL 1X PBS to give a final compound concentration of 0.5 µM and placed in a plate reader at 37 °C. Fluorescence was measured after 10 min of incubation. The propagated error was calculated by adjusting the CES1 and CES2 enzyme activity readings against the compound with no enzyme to remove background noise and non-enzymatic hydrolysis. The ratio was then obtained by dividing the background corrected CES1 value by the CES2 value.

Limit of Detection of CES1 by MCP-Et
100 µL of 1 µM MCP-Me in 1X PBS were added to a 96 well plate before addition of 0.05, 0.025, 0.0125, 0.00625, 0.00313, 0.00156, and 0.00781 units of CES1 or no enzyme in 100 µL 1X PBS to give a final compound concentration of 0.5 µM and placed in a plate reader at 37 °C. Fluorescence was measured after 10 min of incubation and repeated three times. Data were fit and analyzed using GraphPad Prism 10 (GraphPad Software, Boston, MA).

Michaelis-Menten kinetics of MCP-Et with CES1 and CES2. Michalis-Menten kinetics were performed by adapting previously reported procedures.[13,14] To allow for quantification and to correct for photobleaching, 20 µM stock of MOF serial diluted in half six times in 1X PBS (Fisher) to produce a range of 20 µM to 0.78125 µM solutions and 200 µL of each solution were pipetted into a 96 well plate. Substrate solutions (MCP-Et) were prepared by serially diluting a 100 µM stock solution in half seven times in 1X PBS to produce a range of 100 µM to 1.56 µM and 100 µL of each solution was pipetted into a 96 well plate. To begin the experiment, 100 µL of CES1 or CES2 in 1X PBS were added to produce a final concentration of 2.5 units/mL of enzyme in each well and the plate was immediately placed in the plate reader to begin the experiment. Fluorescence was then measured every 10 sec for 30 min using the green filter cube. This was repeated in triplicate. Data were fit and analyzed using GraphPad Prism 10.

HepG2 cell culture conditions. HepG2 cell line was a gift from Prof. Bryan C. Dickinson (University of Chicago) and was maintained in DMEM/High Glucose (10% FBS, 1X Anti-Anti, Glutamax, sodium pyruvate, Gibco or Corning brand, Fisher) with 10% FBS (Benchmark line, GeminiBio, West Sacramento, CA) at 37 °C and 5% CO_2. Cells were used for less than 30 passages for all experiments.[8]

THP-1 cell culture conditions. THP-1 cells are monocytic leukemia cells and when differentiated with phorbol-12-myristate-13-acetate (PMA) they become adherent and display an M0 macrophage phenotype (THP-1 macrophages).[15] Scrambled and CES1-knockdown shRNA THP-1 cell line was a gift from Prof. Matthew K. Ross (Mississippi State University)[15,16] and were maintained in suspension with RPMI-1640 medium (10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 0.05 mM 2-mercaptoethanol, 1X Anti-Anti, ATCC brand) puromycin hydrochloride was also added to the complete growth media at 5 µg/mL final concentration to ensure maintenance of the lentiviral-transduced knockdown cells.[15] Cells

were cultured at 37°C with 5% CO_2 and used for less than 20 passages for all experiments.

Lysine coating coverslip glass bottom dishes for fluorescence microscopy experiments. Four chamber glass bottom dishes (C4-1.5H-N, Cellvis, Mountain View, CA) were coated with 450 µL poly-D-lysine (Gibco brand, Fisher) per chamber for 1 h at room temperature or overnight at 4 °C, followed by washing with 500 µL of DPBS (Gibco brand, Fisher) twice.

Cell fluorescence imaging of MCP-Et in CES1 and CES2 knockdown HepG2 cells. CES1 and CES2 were knocked down following previously reported and validated procedures.[8] To summarize, HepG2 cells were added in 750 µL of DMEM/High Glucose to each well of a lysine coated four chamber glass bottom dish and incubated overnight to a confluency of 70% to 80%. The next day cells were transfected with 0.7 µg of CES1 shRNA plasmid (target sequence: CGGAATTAACAAGCAGGAGTT, TRCN0000046933,), CES2 shRNA plasmid (target sequence: CAGCAGAATATCGCCCACTTT, TRCN0000046964), or scrambled shRNA plasmid (gift from David Sabatini) using 6 µL Transporter 5 Transfection Reagent (Polysciences) with a final volume of 150 µL in Opti-MEM (Gibco brand, Fisher) in serum and antibiotic-free DMEM/Glucose media according to the manufacture's protocol. After 8-12 h, the media was replaced with DMEM/High Glucose. Following 48 h incubation at 37 °C and 5% CO_2, media was removed, and cells were washed with 500 µL of DPBS. The solution was then replaced with 500 µL of imaging solution (Gibco FluoroBrite DMEM supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4) containing 25 µM MCP-Et with 0.1% DMSO. After 30 min incubation at 37 °C, the solution was removed, the cells were washed with 500 µL of DPBS and 500 µL of fresh imaging solution was placed in each well before immediately imaging the cells. Fluorescence was quantified by measuring total fluorescence signal in the green (MCP-Et) channel over background using the threshold feature of ImageJ. Any oversaturated pixels were excluded in quantification of the fluorescence signal.

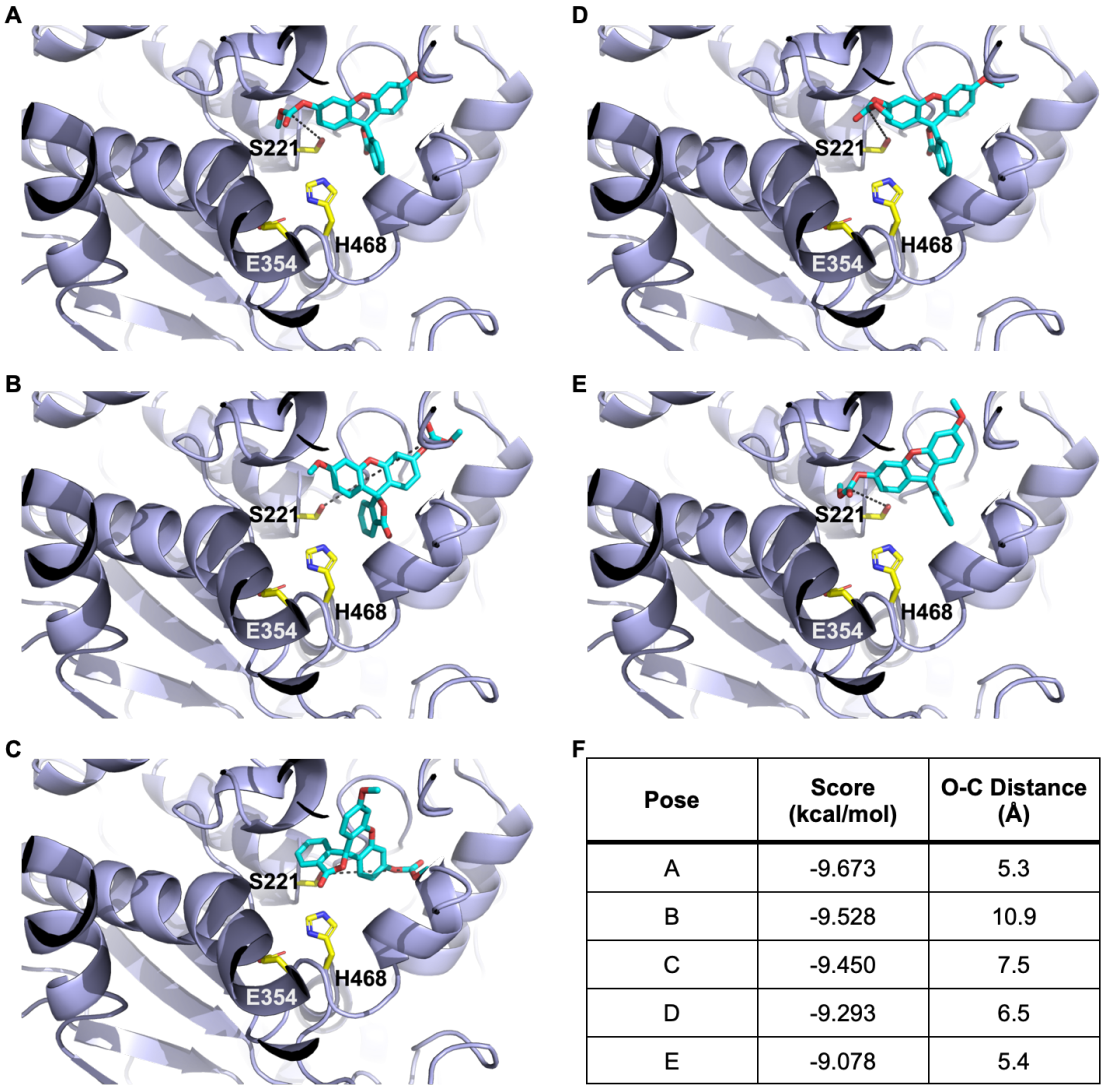
Cell viability studies. To a 96 well tissue culture plate with black side walls (Greiner Bio-One, cat# 655090), 1.5 X 10^5 HepG2 cells in 200 µL of growth medium or 2.1 X 10^5 THP-1 cells in 200 µL of growth medium containing 20 nM phorbol 12-myristate 13-acetate (PMA) were incubated overnight or for 72 h for THP-1 cells at 37 °C and 5% CO_2. At the end of the incubation period the growth media was removed and replaced with 100 µL of 0, 1, 10, or 100 µM MCP-Et in growth media and incubated at 37 °C and 5% CO_2. After 1 h, cells were washed with 100 µL of DPBS and 50 µL of molecular biology grade water (Fisher) was added followed by the addition of 25 µL of freshly prepared ATP-Glo Detection Cocktail (Biotium, cat# 30020). The plate was immediately placed into a plate reader and total luminescence measured (1 second integration time, fiber optic cable, read height = 3.88 mm, autogain set to 70% signal of 0 µM treated cells). Data was analyzed and figures generated using GraphPad Prism 10.

Live cell fluorescence imaging of MCP-Et to study DDIs in HepG2 cells. To each well of a lysine coated four chamber glass bottom dish, HepG2 cells were added in 750 µL of DMEM/High Glucose and incubated overnight to produce 60-70% confluency. The following day, the media was replaced with 500 µL of imaging solution, (Gibco FluoroBrite DMEM supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4) containing either 50 µM troglitazone, 50 µM loperamide, or 0.1% DMSO (control). After incubation at 37 °C for 30 min, the solution was removed and cells were washed with 500 µL of DPBS followed by the addition of 500 µL of 25 µM MCP-Et in fresh imaging solution containing

the appropriate drug or DMSO. After 30 min incubation at 37 °C, the cells were washed with 500 µL of DPBS followed by the addition of 500 µL of fresh imaging solution containing the appropriate drug or DMSO. The cells were then immediately imaged. Fluorescence was quantified by measuring total fluorescence signal in the green (MCP-Et) channel over background using the threshold feature of ImageJ. Any oversaturated pixels were excluded in quantification of the fluorescence signal.

Live cell fluorescence imaging of MCP-Et in CES1 knockdown THP-1 cells. To each well of a lysine coated four chamber glass bottom dish, the THP-1 monocytes (scramble in wells 1 and 2, CES1 knockout in wells 3 and 4) were differentiated to display an M0 macrophage phenotype by incubating in growth medium containing 20 nM phorbol 12-myristate 13-acetate (PMA) for 72 h at 37°C and 5% CO_2. After 72 h, the media was replaced with 500 µL of imaging solution (Gibco FluoroBrite DMEM supplemented with 20 mM HEPES, pH 7.4) containing 25 µM MCP-Et with 0.1% DMSO. After 30 min incubation at 37 °C, the solution was removed, cells washed with 500 µL of DPBS, and 500 µL of fresh imaging solution was placed in each well before immediately imaging the cells. Fluorescence was quantified by measuring total fluorescence signal in the green (MCP-Et) channel over background using the threshold feature of ImageJ. Any oversaturated pixels were excluded in quantification of the fluorescence signal. CES1 knock down was previously validated by Prof. Matthew K. Ross at Mississippi State University.[15]

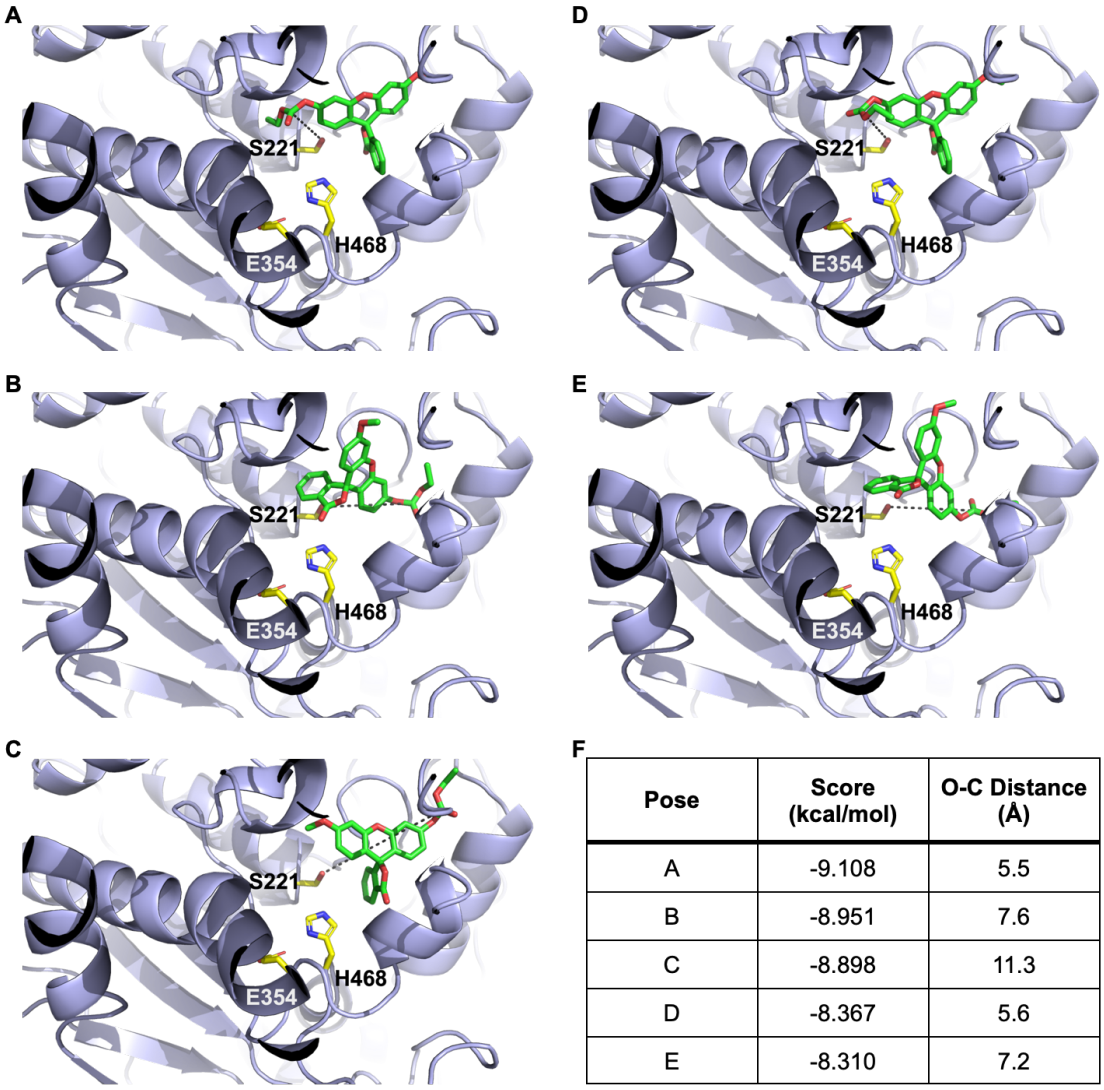
Live cell fluorescence imaging of MCP-Et with inhibitors and drugs in THP-1 cells. In each well of a lysine coated four chamber glass bottom dish, THP-1 monocytes were differentiated to display an M0 macrophage phenotype by incubating in growth medium containing 20 nM phorbol 12-myristate 13-acetate (PMA) for 72 h at 37°C and 5% CO_2. After 72 h, the media was replaced with 500 µL of imaging solution (Gibco FluoroBrite DMEM supplemented with 20 mM HEPES, pH 7.4) containing either 100 µM bis(p-nitrophenyl) phosphate (BNPP), 50 µM troglitazone, 50 µM loperamide, or 0.1% DMSO (control). After incubation at 37 °C for 30 min, the solution was removed and cells were washed with 500 µL of DPBS followed by the addition of 500 µL of 25 µM MCP-Et in fresh imaging solution containing the appropriate compound or DMSO. After 30 min incubation at 37 °C, the cells were washed with 500 µL of DPBS followed by the addition of 500 µL of fresh imaging solution containing the appropriate compound or DMSO. The cells were then immediately imaged. Fluorescence was quantified by measuring total fluorescence signal in the green (MCP-Et) channel over background using the threshold feature of ImageJ. Any oversaturated pixels were excluded in quantification of the fluorescence signal.



F

Pose	Score (kcal/mol)	O-C Distance (Å)
A	-9.673	5.3
B	-9.528	10.9
C	-9.450	7.5
D	-9.293	6.5
E	-9.078	5.4

Figure S1. A-E. Top five scoring molecular docking poses of MCP-Me (blue) with CES1 (PDB ID: 1DR0; purple). The side chains of the catalytic triad (Ser221, E354, and H468) are highlighted in yellow. F. Table of score and distance between the Ser221 oxyanion and the carbonyl of MCP-Me (black dashed line in A-E).



F

Pose	Score (kcal/mol)	O-C Distance (Å)
A	-9.108	5.5
B	-8.951	7.6
C	-8.898	11.3
D	-8.367	5.6
E	-8.310	7.2

Figure S2. A-E. Top five scoring molecular docking poses of MCP-Et (green) with CES1 (PDB ID: 1DR0; purple). The side chains of the catalytic triad (Ser221, E354, and H468) are highlighted in yellow. F. Table of score and distance between the Ser221 oxyanion and the carbonyl of MCP-Et (black dashed line in A-E).

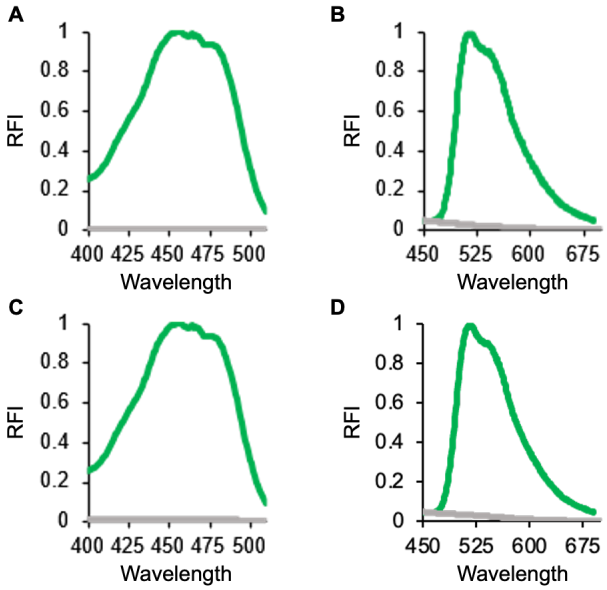


Figure S3. A. Excitation and B. emission fluorescence spectra of MCP-Me (gray) and C. excitation and D. emission fluorescence spectra of MCP-Et (gray) compared to MOF (green).

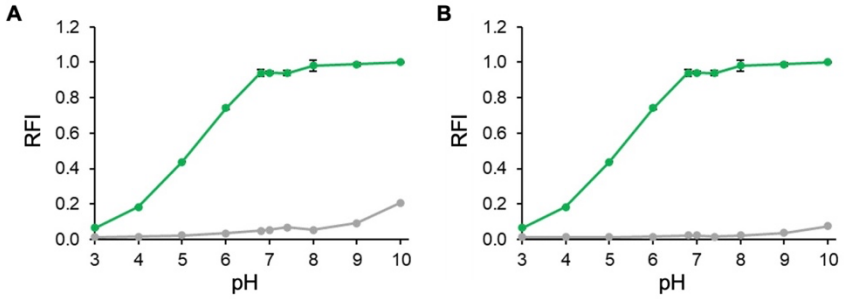


Figure S4. Solution stability of A. MCP-Me and B. MCP-Et (gray) compared to MOF (green) at variable pH (3.0-10.0). RFI = Relative Fluorescence Intensity to MOF at pH 10. Error bars are +/- std. dev. (n = 3).

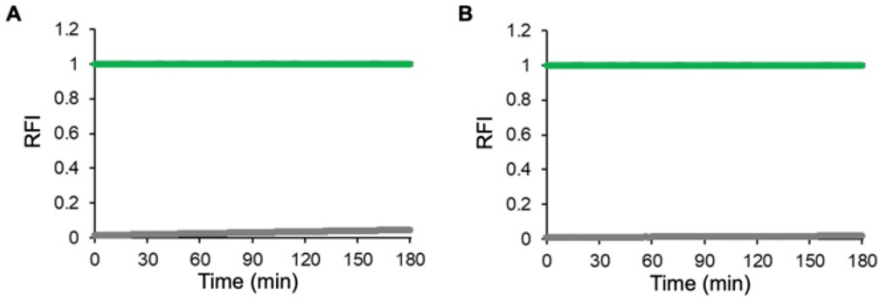


Figure S5. Solution stability of A. MCP-Me and B. MCP-Et (gray) compared to MOF (green) in 1X PBS, pH 7.4. RFI = Relative Fluorescence Intensity to MOF at same time point. Error bars are +/- std. dev. (n = 3).

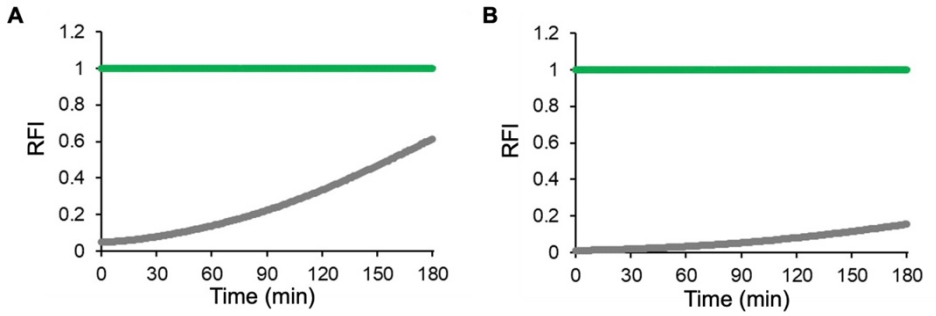


Figure S6. Solution stability of A. MCP-Me and B. MCP-Et (gray) compared to MOF (green) in a complex solution (FluoroBrite DMEM supplemented with 20 mM HEPES, pH 7.4) RFI = Relative Fluorescence Intensity to MOF at same time point. Error bars are +/- std. dev. (n = 3).

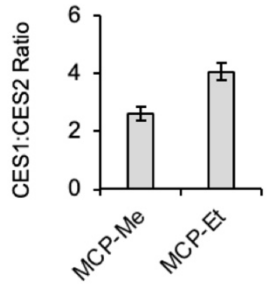
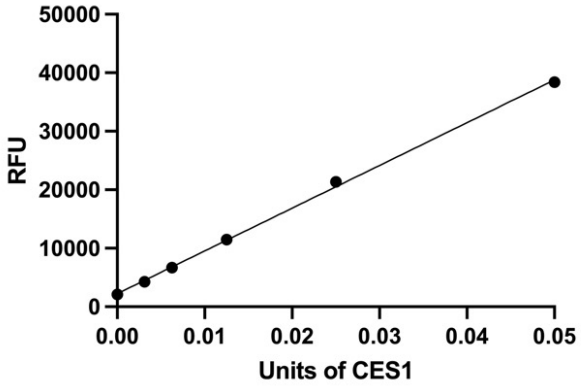


Figure S7. Ratio of hydrolysis of 0.5 µM MCP-Me and MCP-Et by 0.25 units of CES1 and CES2 after 10 min. Error bars are +/- std. dev. (n = 3).



	Best-fit value	95% Confidence Interval
Slope	730872	717175 to 744568
Y-intercept	2237	1915 to 2560
X-intercept	-0.003061	-0.003551 to -0.002585

Goodness of Fit	
R squared	0.9988
Sy.x	470.3

Figure S8. Limit of detection study and linear fit results. RFU = Relative Fluorescence Units. Error bars are +/- std. dev. (n = 3).

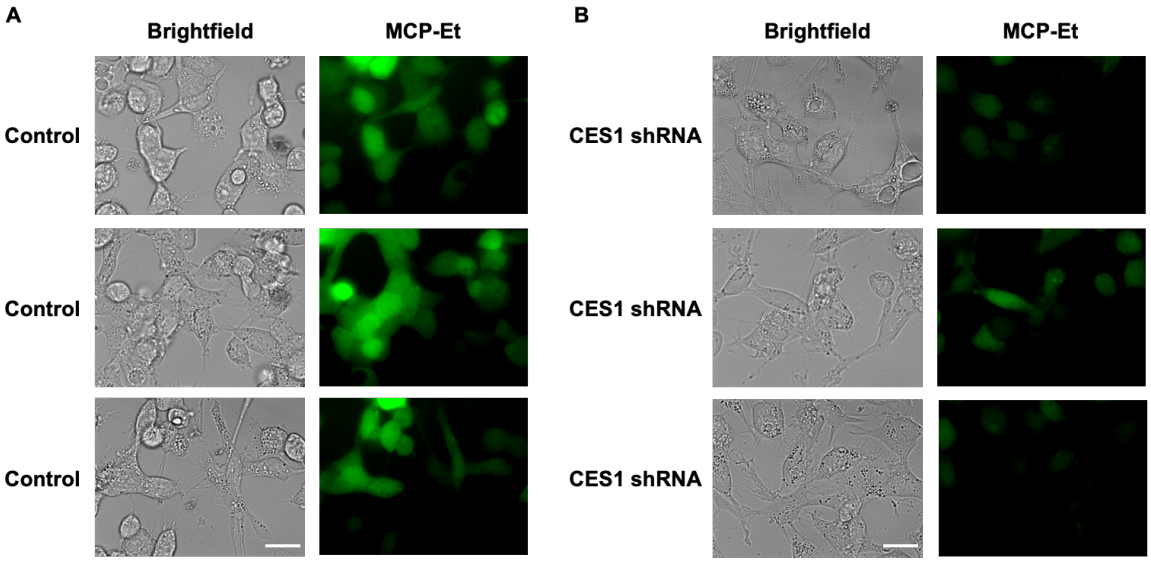


Figure S9. All images used in analysis of fluorescence imaging of MCP-Et in live HepG2 cells with CES1 knockdown. A. Cells were transfected with scrambled shRNA vector (control) or B. a vector generating shRNA targeting CES1 (CES1 shRNA) for 48 h before loading with 25 μM MCP-Et for 30 min and imaged. Scalebar = 20 μm.

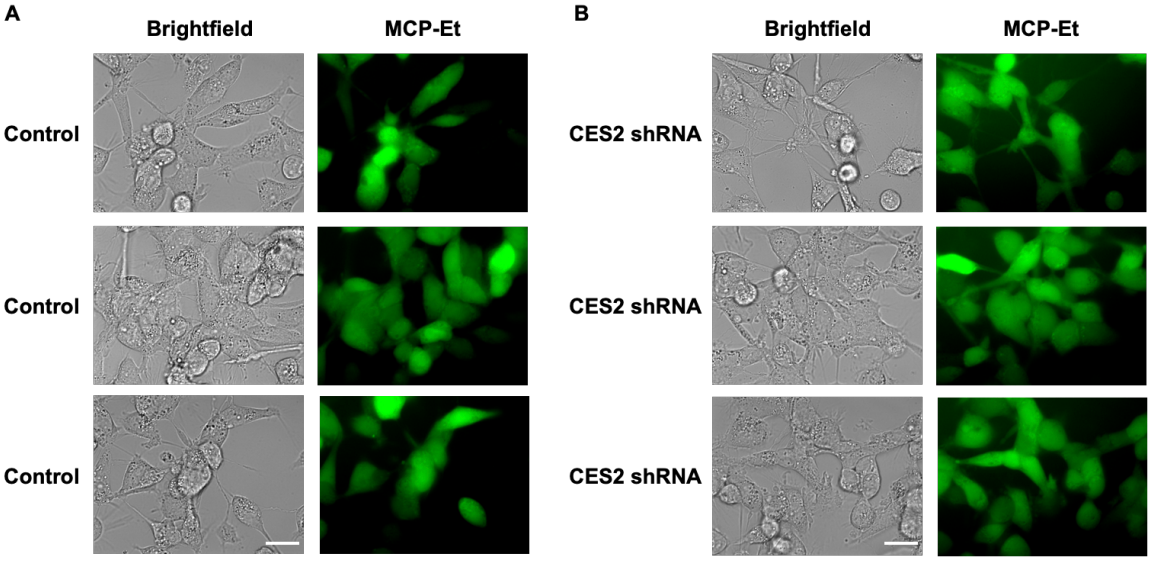


Figure S10. All images used in analysis of fluorescence imaging of MCP-Et in live HepG2 cells with CES1 knockdown. A. Cells were transfected with scrambled shRNA vector (control) or B. a vector generating shRNA targeting CES2 (CES2 shRNA) for 48 h before loading with 25 μM MCP-Et for 30 min and imaged. Scalebar = 20 μm.

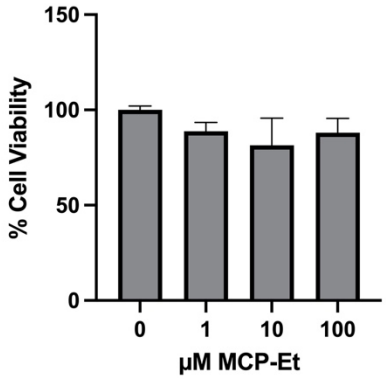


Figure S11. Cell viability of HepG2 cells treated with 0, 1, 10, or 100 μM of MCP-Et for 1 hour.

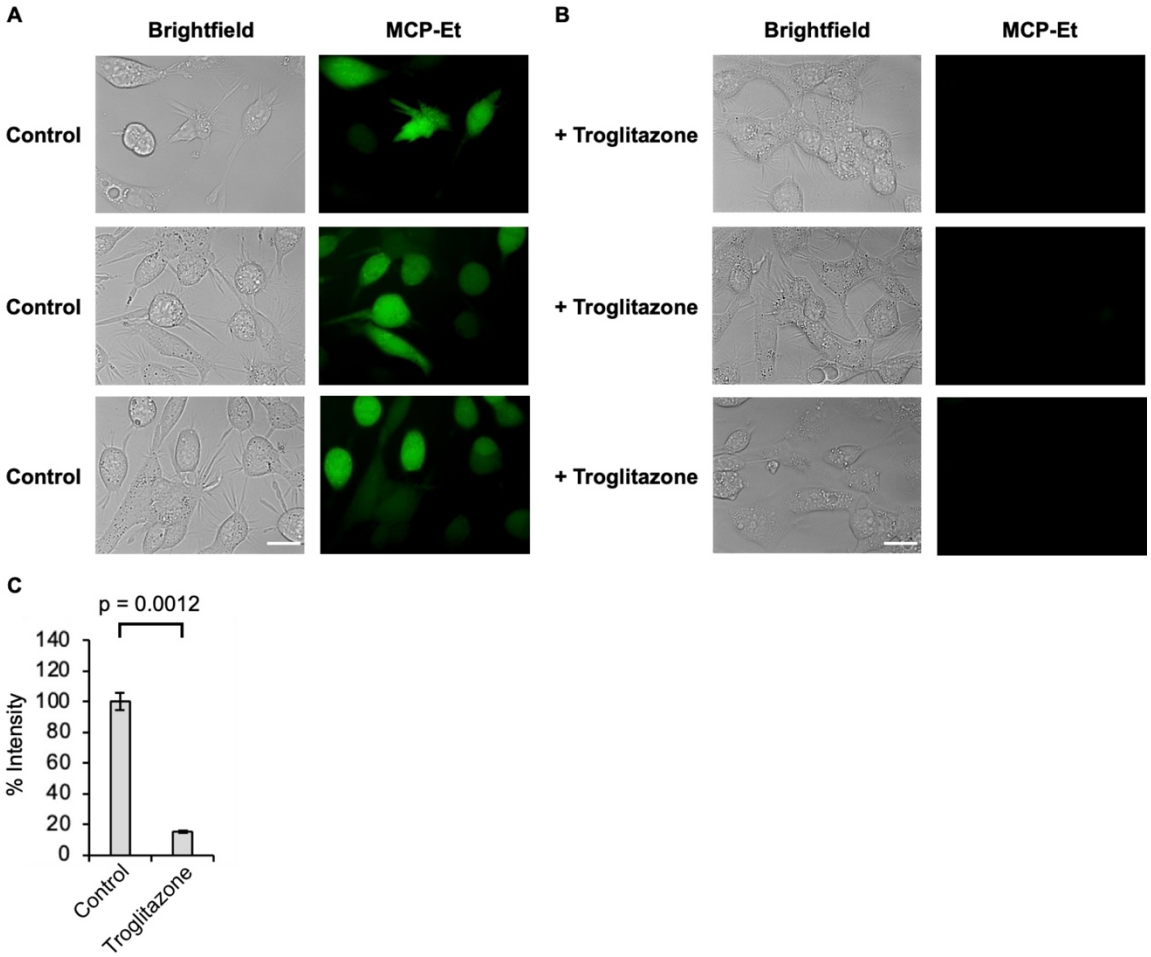


Figure S12. All images used in analysis of fluorescence imaging of MCP-Et in live HepG2 cells with troglitazone. Cells were treated with A. DMSO (control) or B. 50 μM troglitazone for 30 min before loading with 25 μM MCP-Et for 30 min and imaged. C. Quantification of fluorescence signal of experiment described in A and B. Error bars are ± std. dev. (n = 3). Scalebar = 20 μm.

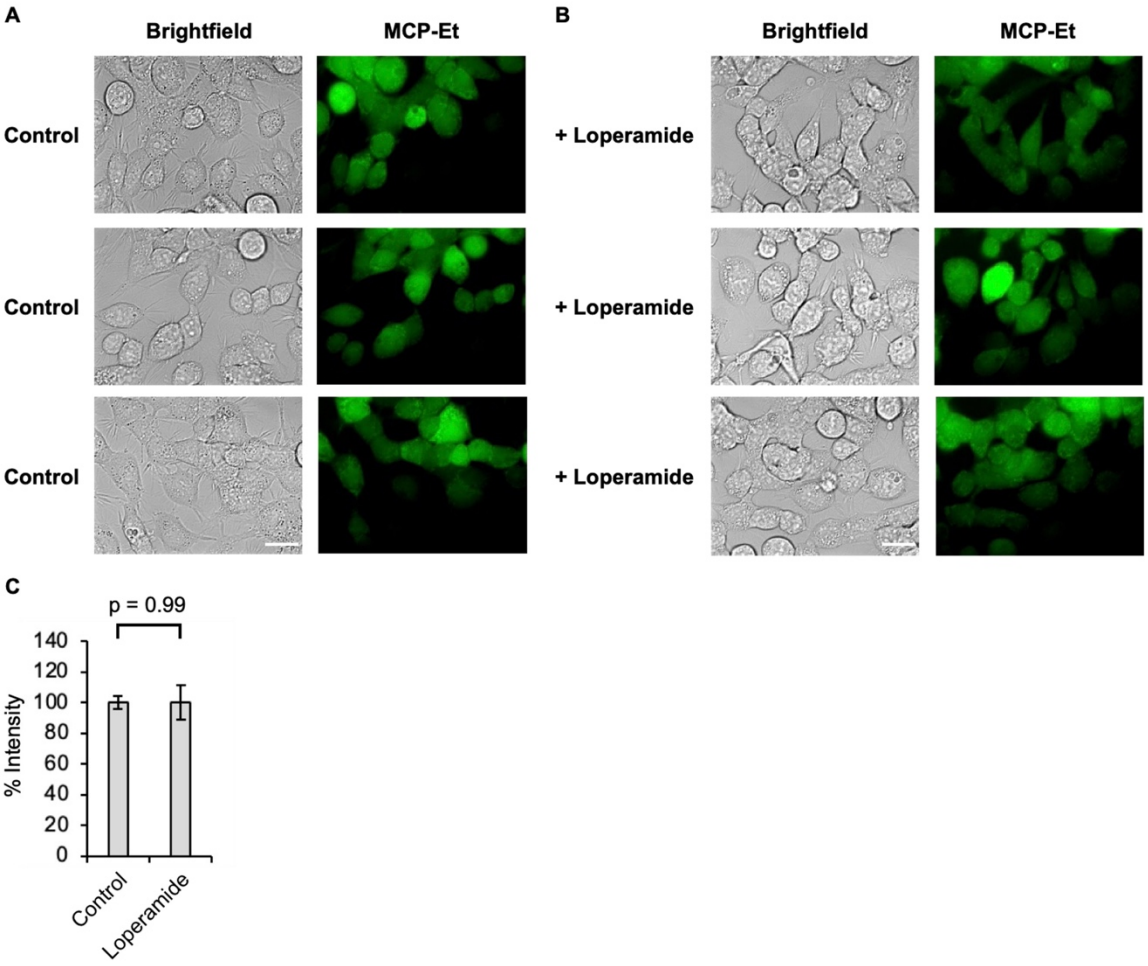


Figure S13. All images used in analysis of fluorescence imaging of MCP-Et in live HepG2 cells with loperamide. Cells were treated with A. DMSO (control) or B. 50 μM loperamide for 30 min before loading with 25 μM MCP-Et for 30 min and imaged. C. Quantification of fluorescence signal of experiment described in A and B. Error bars are ± std. dev. (n = 3). Scalebar = 20 μm.

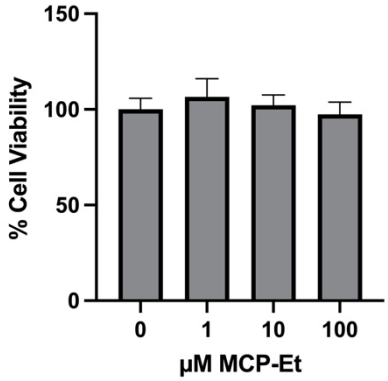


Figure S14. Cell viability of THP-1 macrophages treated with 0, 1, 10, or 100 μM of MCP-Et for 1 hour.

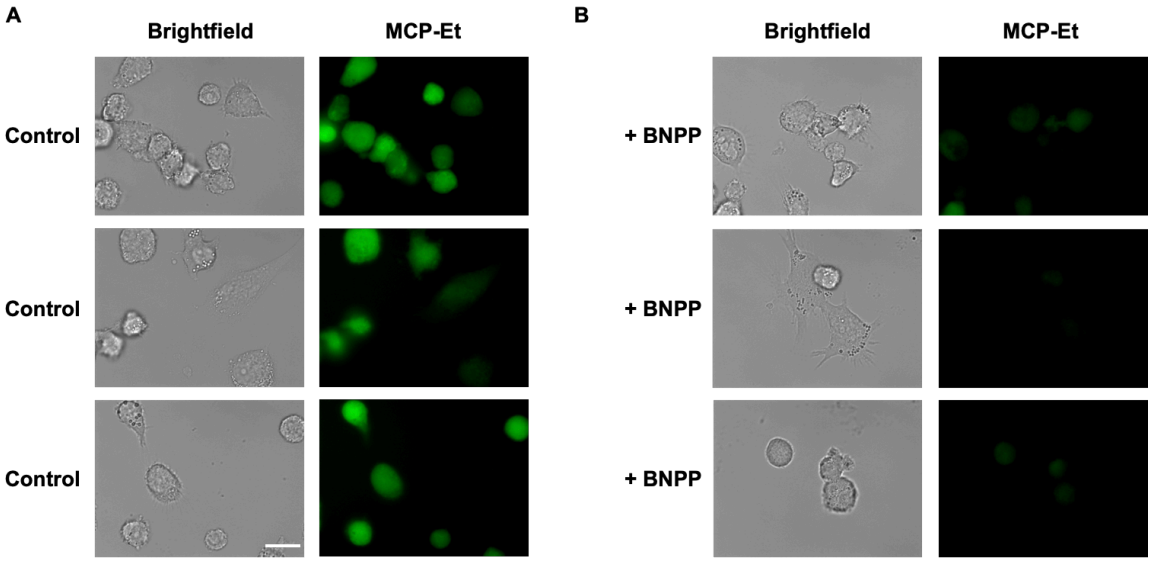


Figure S15. All images used in analysis of fluorescence imaging of MCP-Et in live THP-1 macorphages cells with BNPP. Cells were treated with A. DMSO (control) or B. 100 μM BNPP for 30 min before loading with 25 μM MCP-Et for 30 min and imaged. Scalebar = 20 μm.

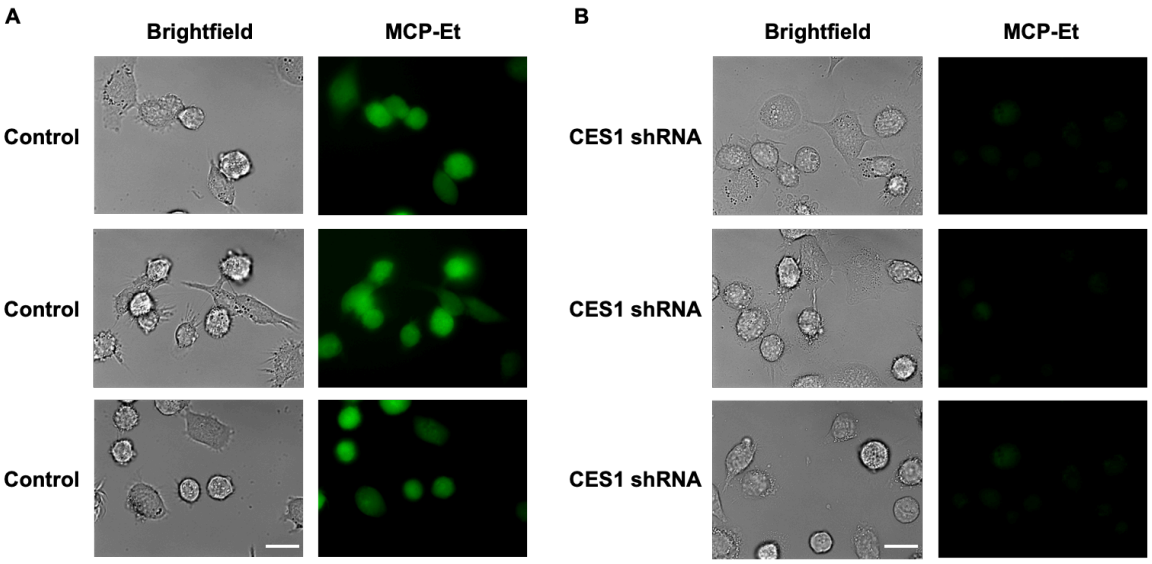


Figure S16. All images used in analysis of fluorescence imaging of MCP-Et in live THP-1 macrophages with CES1 knockdown. A. THP-1 cells expressing scrambled shRNA vector (control) or B. expressing shRNA targeting CES1 (CES1 shRNA) were treated with PMA for 72 h before loading with 25 μM MCP-Et for 30 min and imaged. Scalebar = 20 μm.

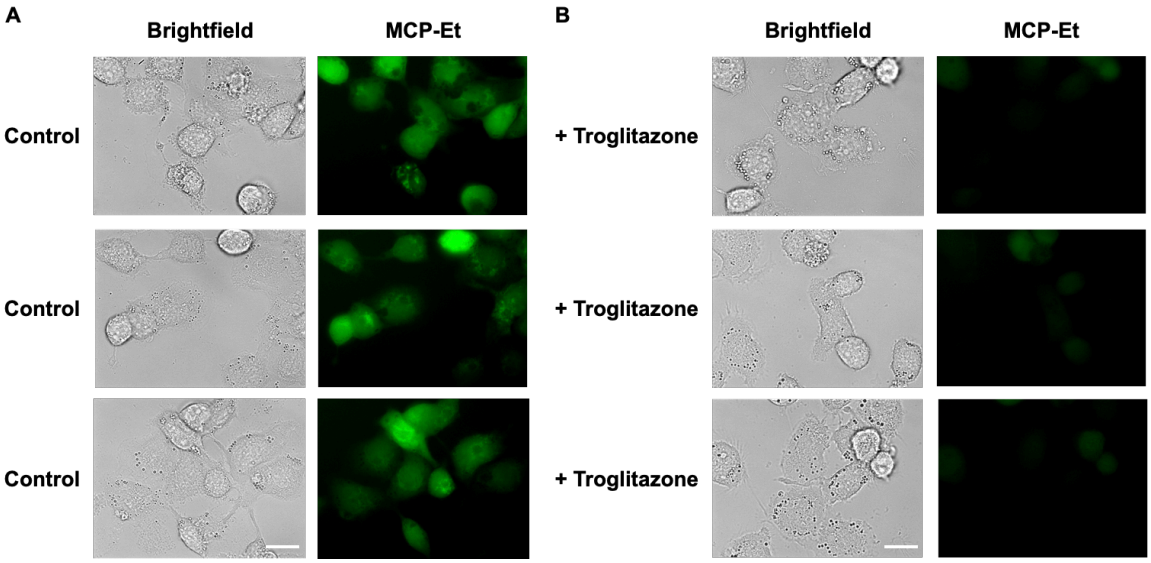


Figure S17. All images used in analysis of fluorescence imaging of MCP-Et in live THP-1 macrophages with troglitazone. Cells were treated with A. DMSO (control) or B. 50 μM troglitazone for 30 min before loading with 25 μM MCP-Et for 30 min and imaged. Scalebar = 20 μm.

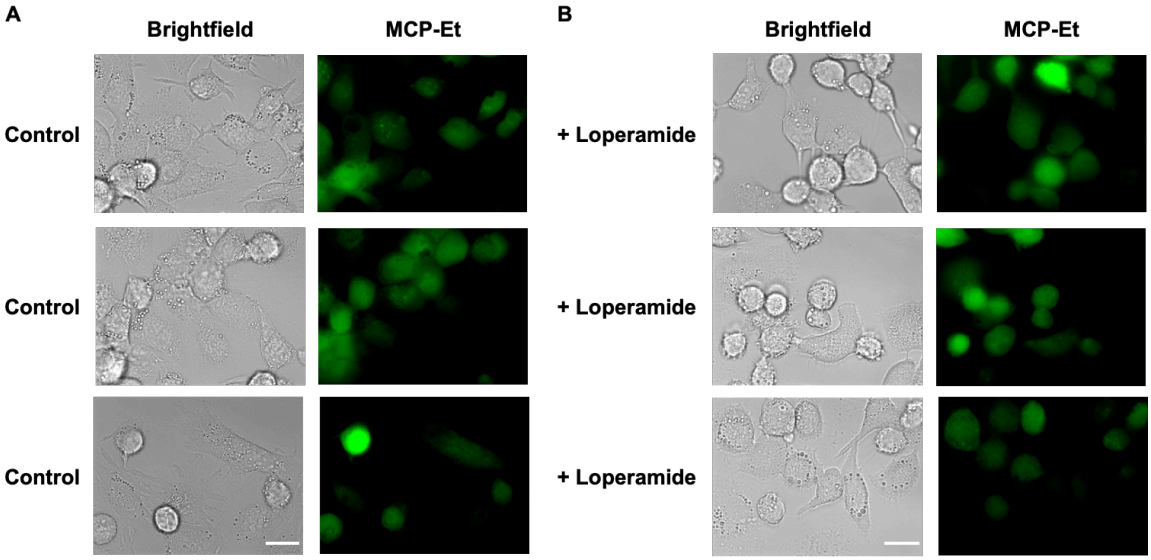
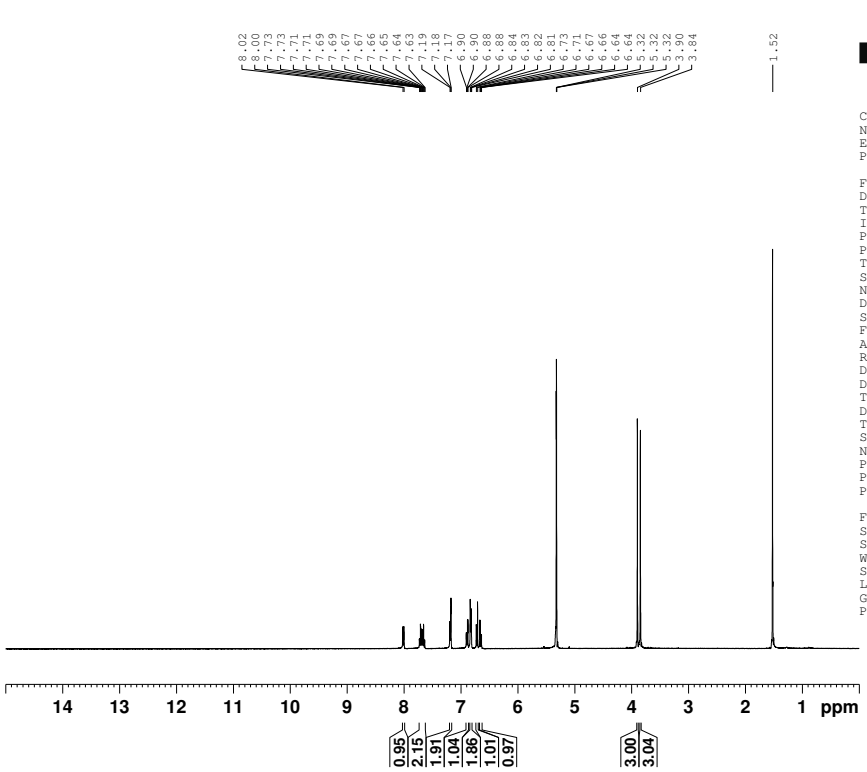
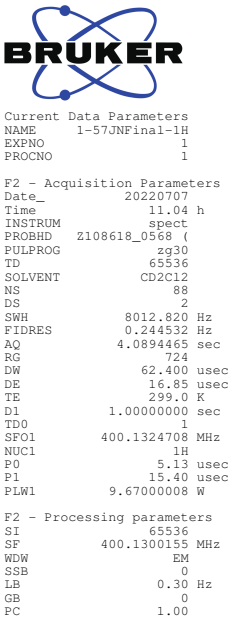


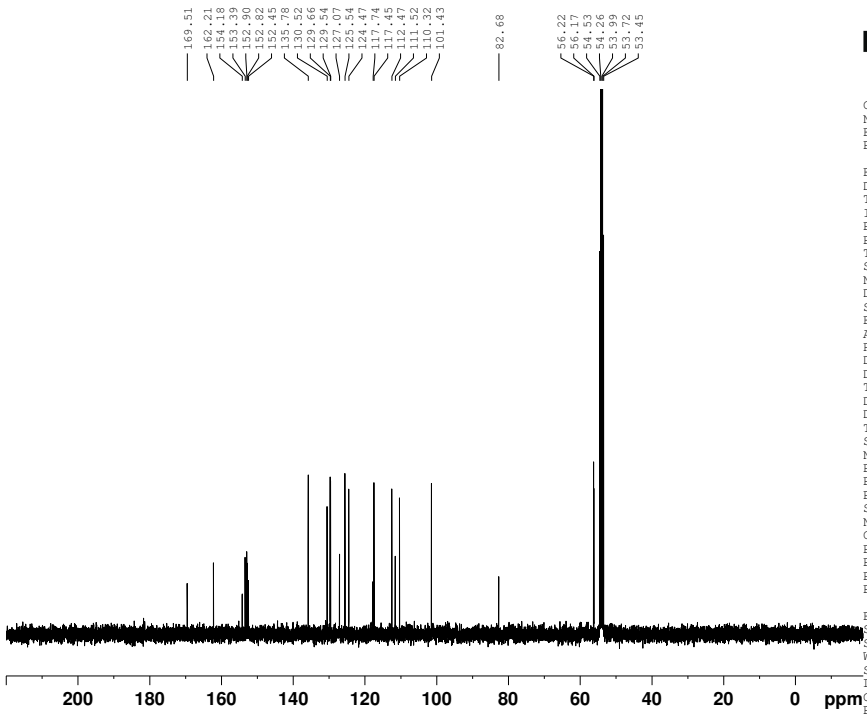
Figure S18. All images used in analysis of fluorescence imaging of MCP-Et in live THP-1 macrophages with loperamide. Cells were treated with A. DMSO (control) or B. 50 μM loperamide for 30 min before loading with 25 μM MCP-Et for 30 min and imaged. Scalebar = 20 μm.

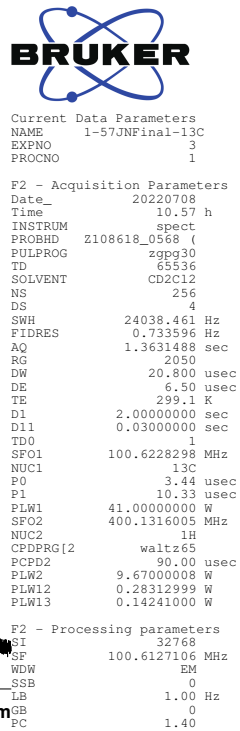
Supplementary Note 1. Chemical Characterization data for MCP-Me



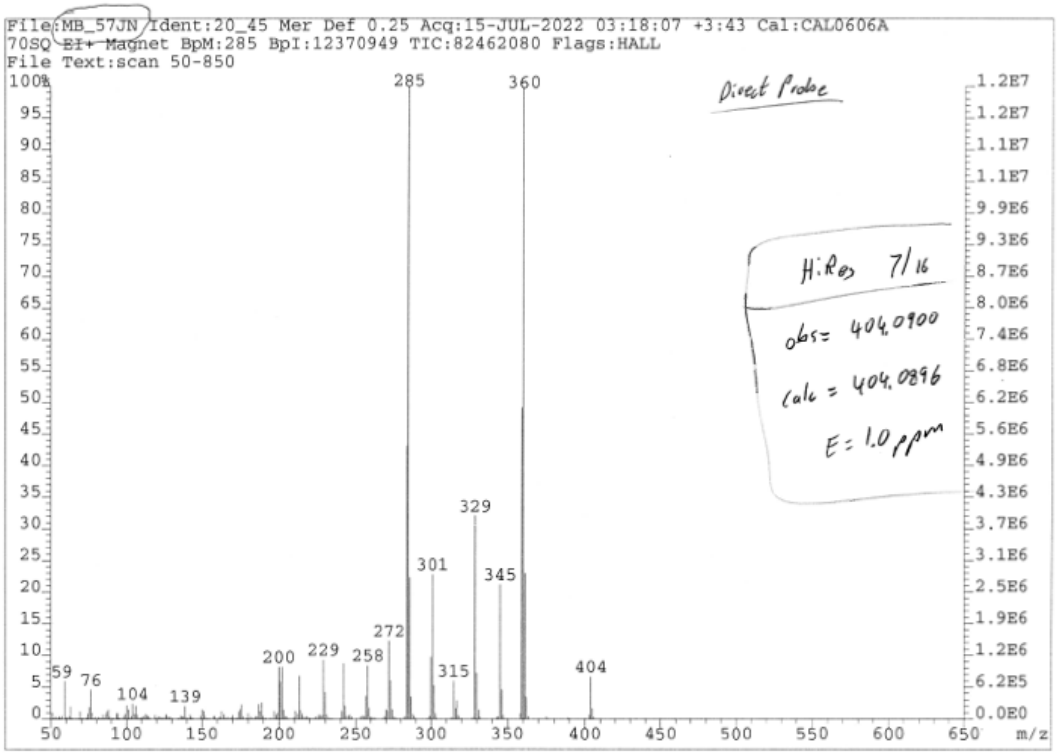


^{1}H NMR of MCP-Me.



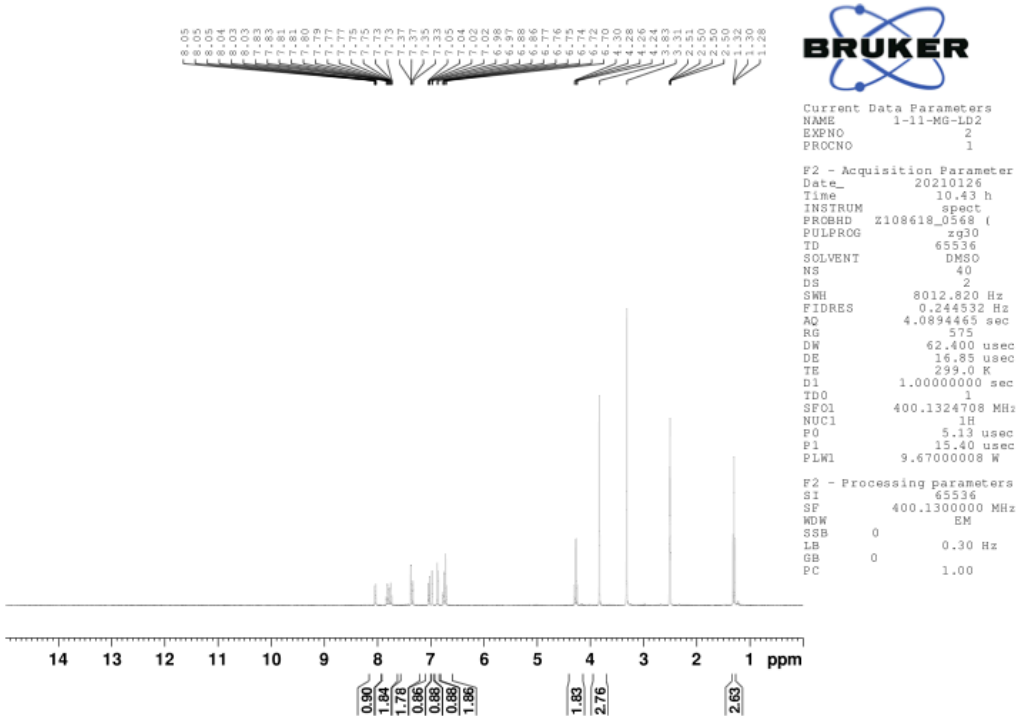


^{13}C NMR of MCP-Me.

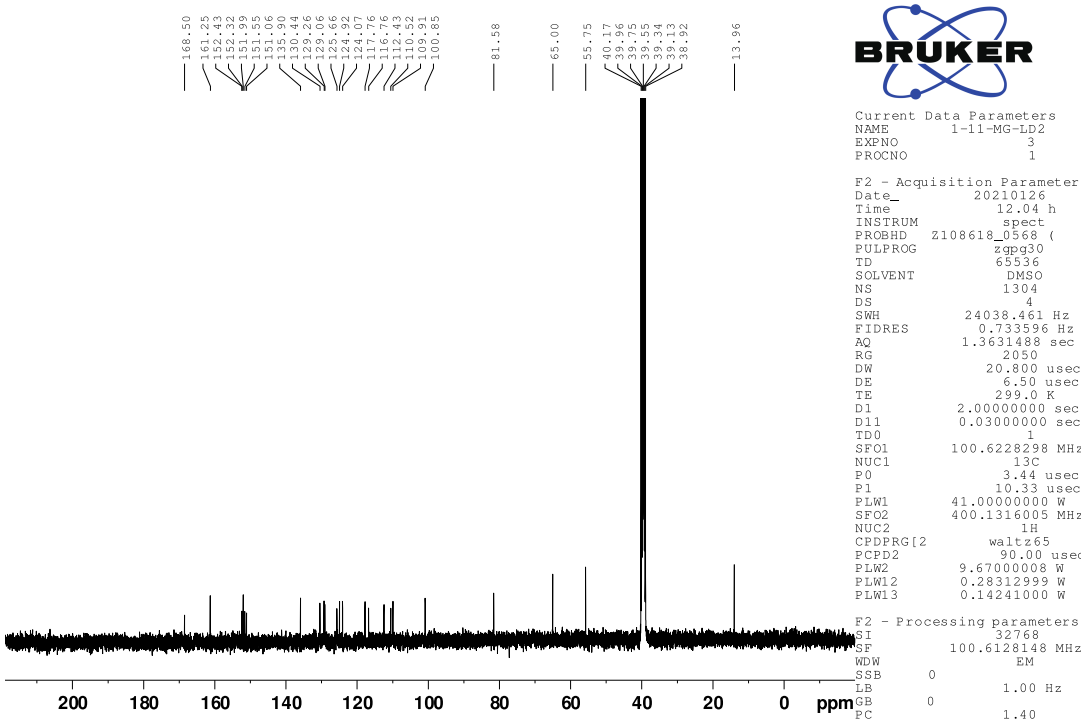


HRA-MS of MCP-Me.

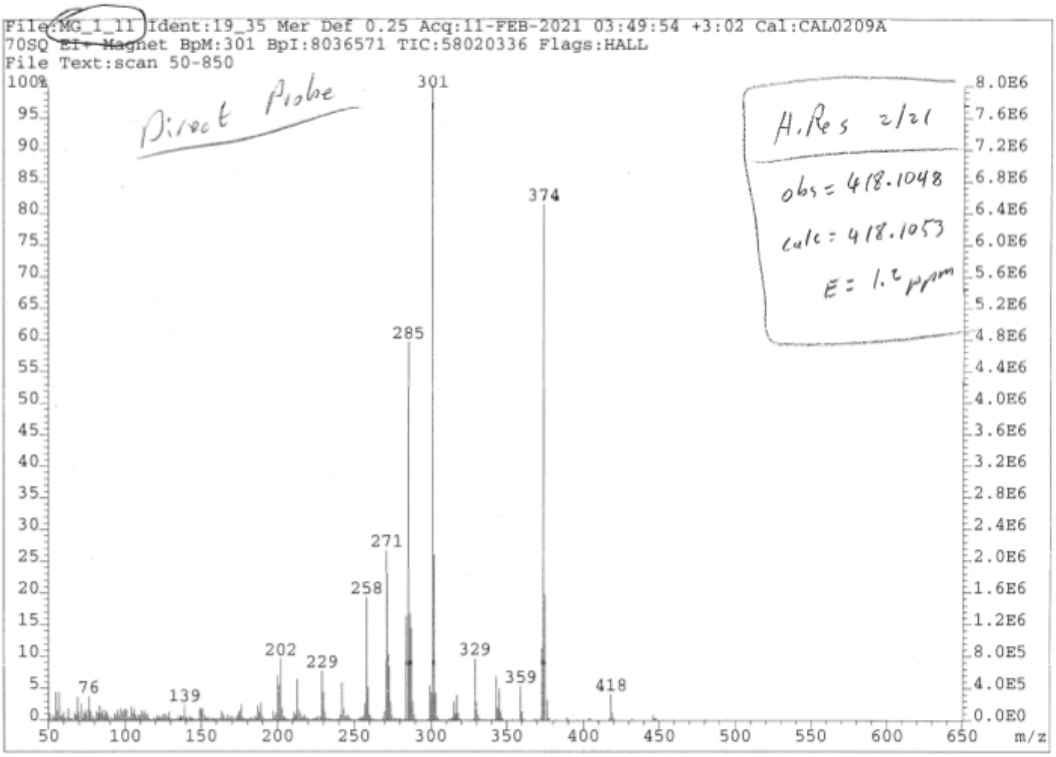
Supplementary Note 2. Chemical Characterization data for MCP-Et



^{1}H NMR of MCP-Et.



^{13}C NMR of MCP-Et.



HRA-MS of MCP-Et.

References

1 J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona, *Nat. Methods*, 2012, **9**, 676–682.

2 J. Eberhardt, D. Santos-Martins, A. F. Tillack and S. Forli, *J. Chem. Inf. Model.*, 2021, **61**, 3891–3898.

3 O. Trott and A. J. Olson, *J. Comput. Chem.*, 2009, **31**, 455–461.

4 Y. Kochnev, E. Hellemann, K. C. Cassidy and J. D. Durrant, *Bioinformatics*, 2020, **36**, 4513–4515.

5 S. Bencharit, C. C. Edwards, C. L. Morton, E. L. Howard-Williams, P. Kuhn, P. M. Potter and M. R. Redinbo, *J. Mol. Biol.*, 2006, **363**, 201–214.

6 M. D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeerschd, E. Zurek and G. R. Hutchison, *J. Cheminformatics*, 2012, **4**, 1–17.

7 J. Zhang, Y.-Q. Sun, J. Liu, Y. Shi and W. Guo, *Chem. Commun.*, 2013, **49**, 11305–11307.

8 A. Singh, M. Gao, C. J. Karns, T. P. Spidle and M. W. Beck, *ChemBioChem*, 2022, **23**, e202200069.

9 M. Martineau, A. Somasundaram, J. B. Grimm, T. D. Gruber, D. Choquet, J. W. Taraska, L. D. Lavis and D. Perrais, *Nat. Commun.*, 2017, **8**, 1412.

10 A. Singh, M. Gao and M. W. Beck, *RSC Med. Chem.*, 2021, **12**, 1142–1153.

11 D. L. Kroetz, F. J. Gonzalez and O. W. McBride, *Biochemistry*, 1993, **32**, 11606–11617.

12 V. Arena de Souza, D. J. Scott, J. E. Nettleship, N. Rahman, M. H. Charlton, M. A. Walsh and R. J. Owens, *PLOS ONE*, 2015, **10**, e0143919.
13 R. J. Johnson, G. C. Hoops, C. J. Savas, Z. Kartje and L. D. Lavis, *J. Chem. Educ.*, 2015, **92**, 385–388.
14 J. Wang, E. T. Williams, J. Bourgea, Y. N. Wong and C. J. Patten, *Drug Metab. Dispos. Biol. Fate Chem.*, 2011, **39**, 1329–1333.
15 J. A. Crow, B. L. Middleton, A. Borazjani, M. J. Hatfield, P. M. Potter and M. K. Ross, *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids*, 2008, **1781**, 643–654.
16 L. C. Mangum, X. Hou, A. Borazjani, J. H. Lee, M. K. Ross and J. A. Crow, *Biochem. J.*, 2018, **475**, 621–642.