Supplementary Material

Tropolone-Induced Effects on the Unfolded Protein Response Pathway and Apoptosis in Myeloma Cells Are Dependent on Iron

Leukemia Research

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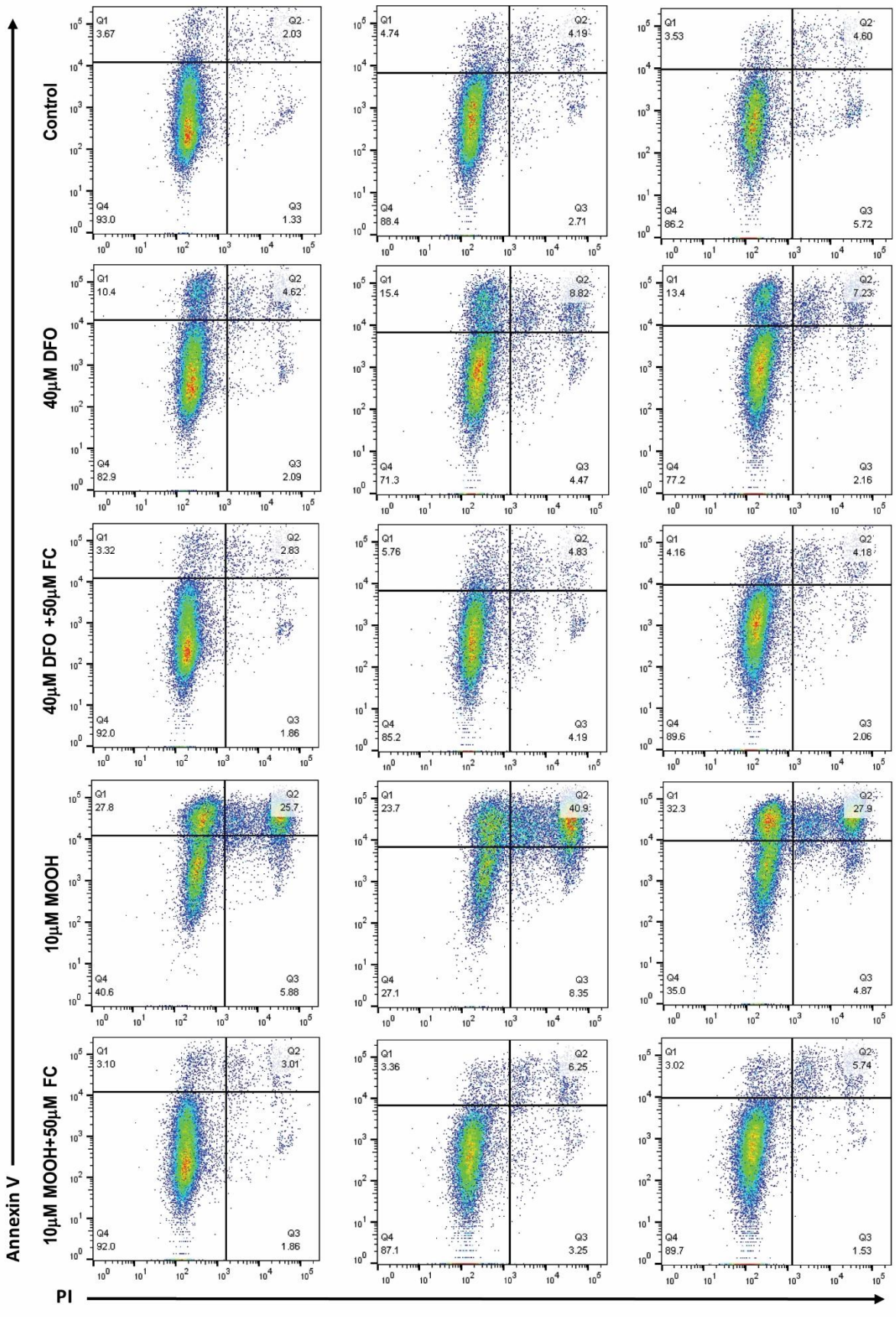
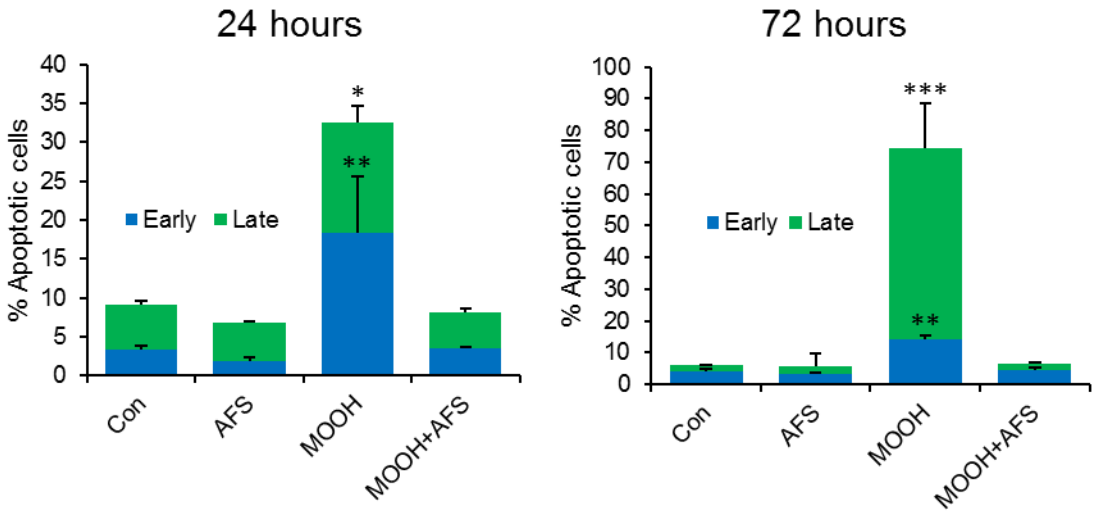
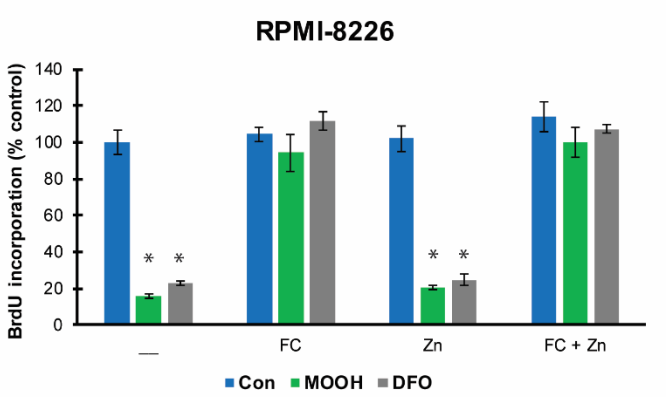

Control
40μM DFO
40μM DFO +50μM FC
10μM MOOH
10μM MOOH+50μM FC
Annexin V
PI
Q1 3.67
Q2 2.03
Q4 93.0
Q3 1.33
Q1 4.74
Q2 4.19
Q4 88.4
Q3 2.71
Q1 3.53
Q2 4.60
Q4 86.2
Q3 5.72
Q1 10.4
Q2 4.62
Q4 82.9
Q3 2.09
Q1 15.4
Q2 8.82
Q4 71.3
Q3 4.47
Q1 13.4
Q2 7.23
Q4 77.2
Q3 2.16
Q1 3.32
Q2 2.83
Q4 92.0
Q3 1.86
Q1 5.76
Q2 4.83
Q4 85.2
Q3 4.19
Q1 4.16
Q2 4.18
Q4 89.6
Q3 2.06
Q1 27.8
Q2 25.7
Q4 40.6
Q3 5.88
Q1 23.7
Q2 40.9
Q4 27.1
Q3 8.35
Q1 32.3
Q2 27.9
Q4 35.0
Q3 4.87
Q1 3.10
Q2 3.01
Q4 92.0
Q3 1.86
Q1 3.36
Q2 6.25
Q4 87.1
Q3 3.25
Q1 3.02
Q2 5.74
Q4 89.7
Q3 1.53

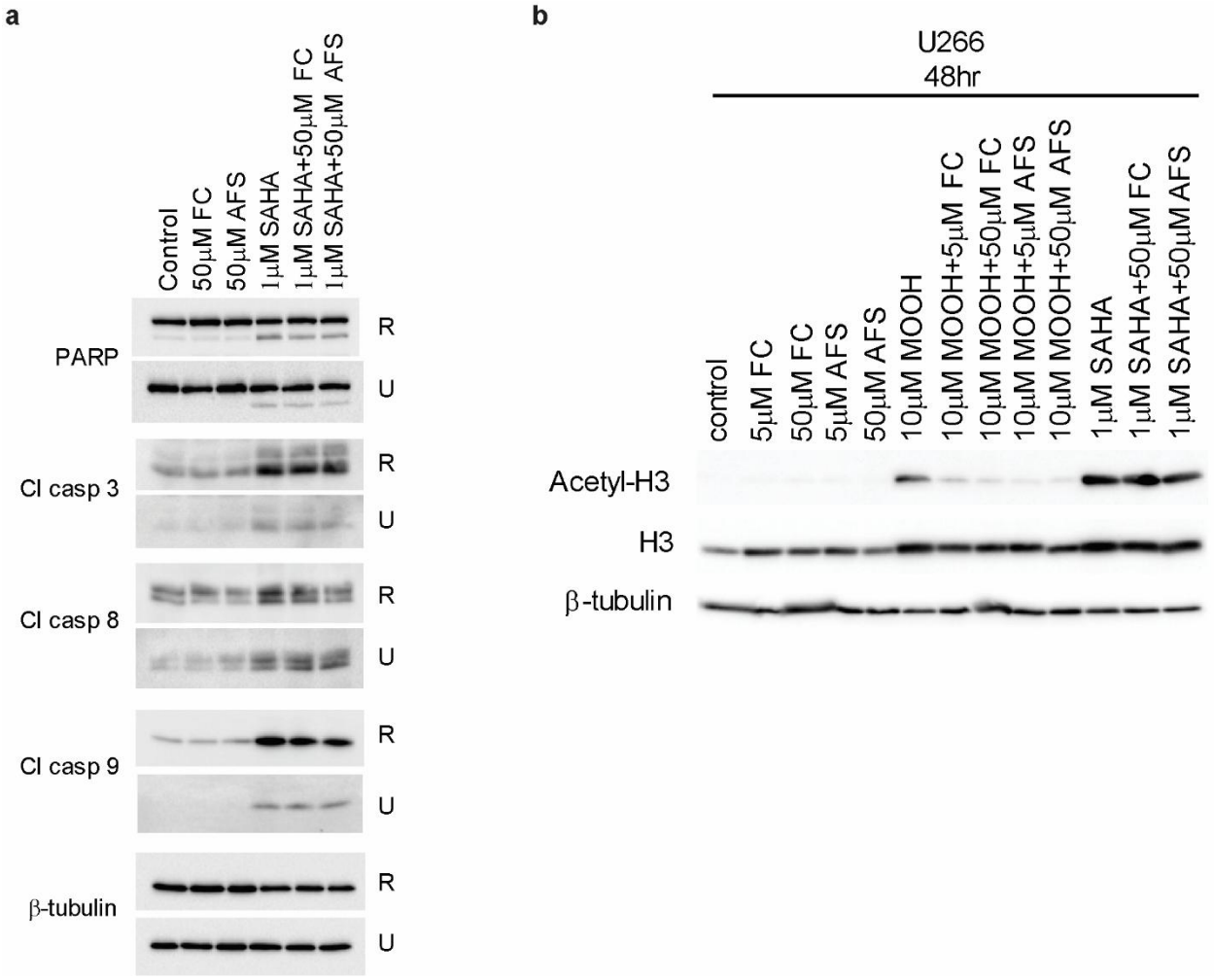
Supplemental Figure 1. Iron abrogates MO-OH-Nap and DFO induced apoptosis in MM cells. Flow diagrams of U266 cells treated for 48 hours with DFO or MO-OH-Nap in the presence and absence of $FeCl_3$ (FC). Apoptosis was measured via flow cytometry using Annexin V (Y-axis) and propidium iodide (X-axis). 10,000 cells per group were recorded. Percentage of cells in each quadrant is shown. Control samples were treated with solvent control (DMSO). N=3.



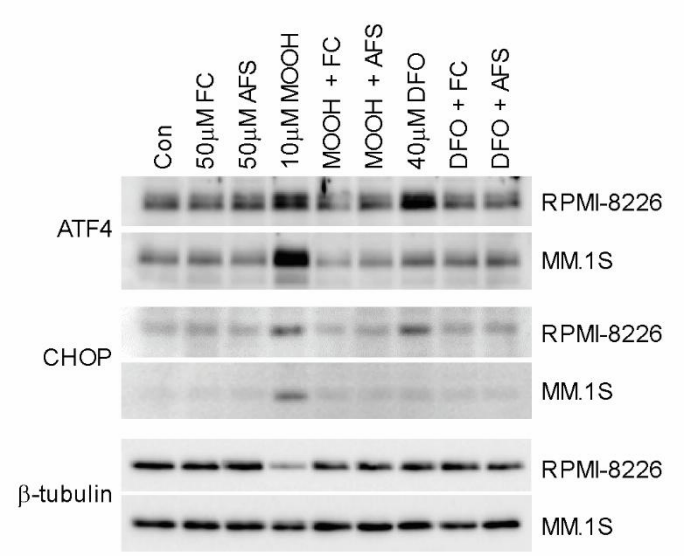
Supplemental Figure 2. Iron abrogates MO-OH-Nap-induced apoptosis in MM cells. Summary of flow data for U266 cells treated for 24 or 72 hours with 10 μM MO-OH-Nap (MOOH) in the presence and absence of 50 μM $FeSO_4$ (AFS). Apoptosis was measured via flow cytometry using Annexin V and propidium iodide. Early apoptotic are defined as AnnexinV+/PI- and late apoptotic are defined as AnnexinV+/PI+. Data are shown as average percentage of early and late apoptotic cells (n=3, * denotes $p<0.01$, ** denotes $p<0.001$, ***denoted $p<0.0001$, per t-test).



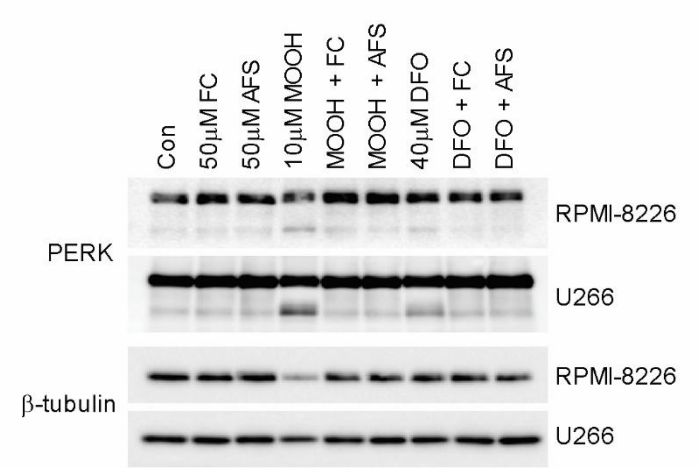
Supplemental Figure 3. The ability for MO-OH-Nap and DFO to inhibit proliferation is independent of zinc. RPMI-8226 cells were treated with DMSO (solvent control), 2.5μM MO-OH-Nap (MOOH), or 40μM DFO in the presence or absence of 50μM $FeCl_3$ (FC) or 50μM $ZnCl_2$ (Zn). BrdU incorporation was measured 48 hours later. (n=3, * denotes $p<0.01$ per t-test)



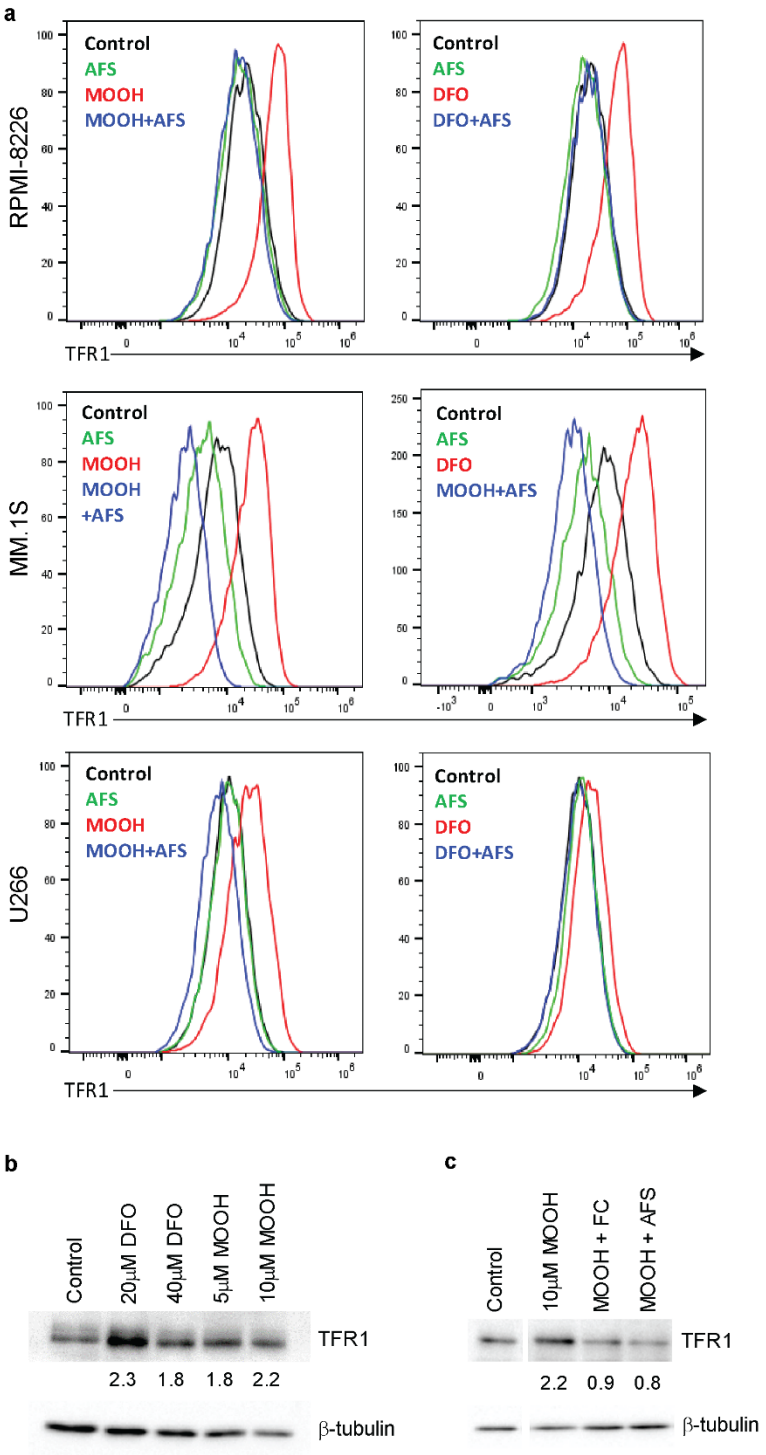
Supplemental Figure 4. Co-incubation with iron does not alter SAHA-induced apoptosis or changes in histone acetylation. a) RPMI-8226 (R) and U266 (U) cells were treated for 48 hours with either solvent control (DMSO) or combinations of $FeCl_3$ (FC), $FeSO_4$ (AFS) and SAHA. Protein levels of PARP and cleaved caspases (3, 8, and 9) were assessed via immunoblot. β-tubulin served as a loading control. **b)** U266 were treated for 48 hours with DMSO or a combination of FC, AFS, MO-OH-Nap (MOOH) or SAHA. Protein levels of acetylated histone H3 and total histone H3 were measured via immunoblot. β-tubulin served as a loading control.



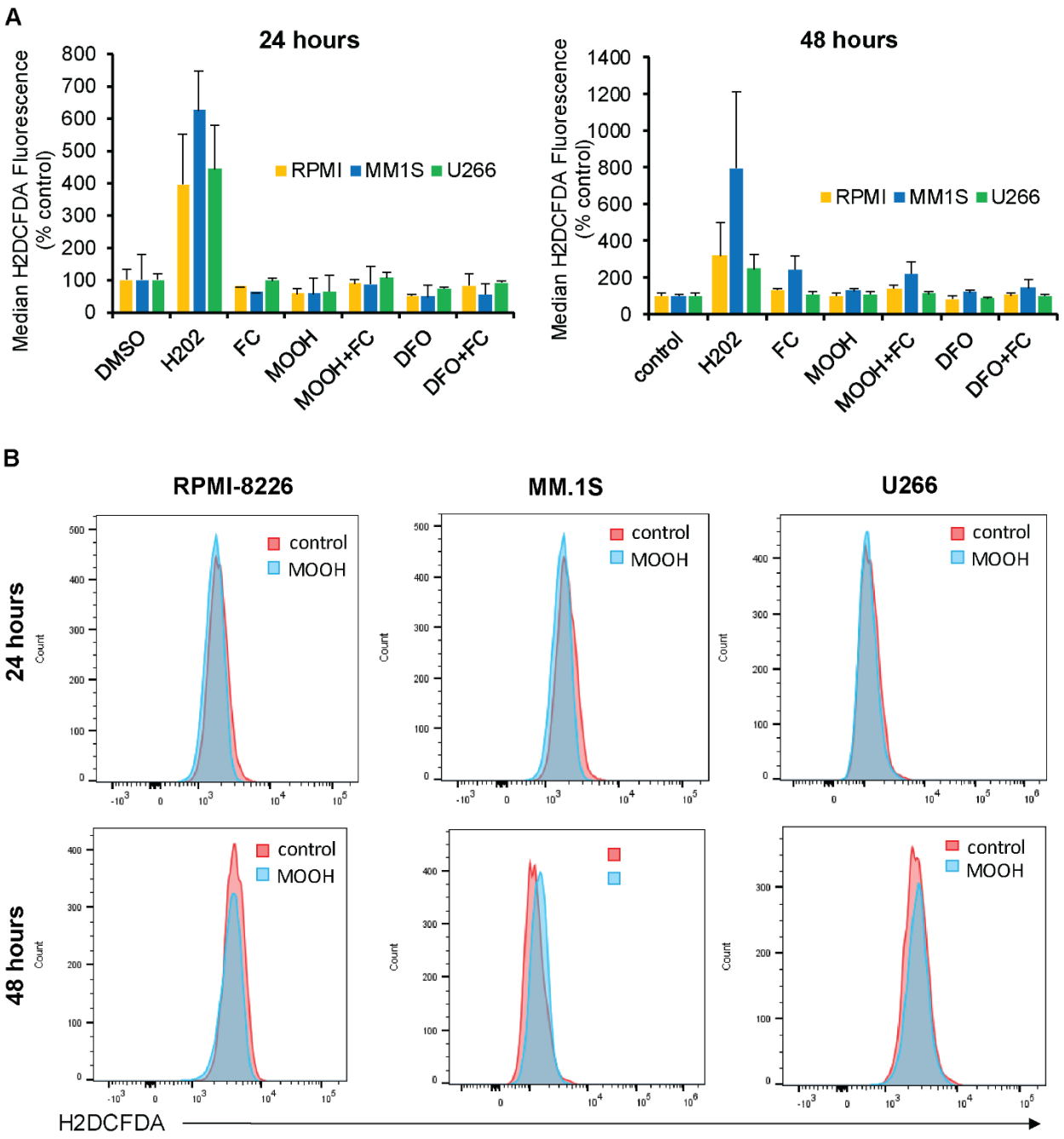
Supplmental Figure 5. Co-incubation with iron reversed the MOOH and DFO induced upregulation of ATF4 and CHOP. RPMI-8226 and MM.1S cells were treated for 48 hours with DMSO, MO-OH-Nap or DFO in the presence or absence of FC and AFS. Protein levels of ATF4, CHOP and β-tubulin were measured by immunoblot. Immunoblots are representative of three independent experiments.



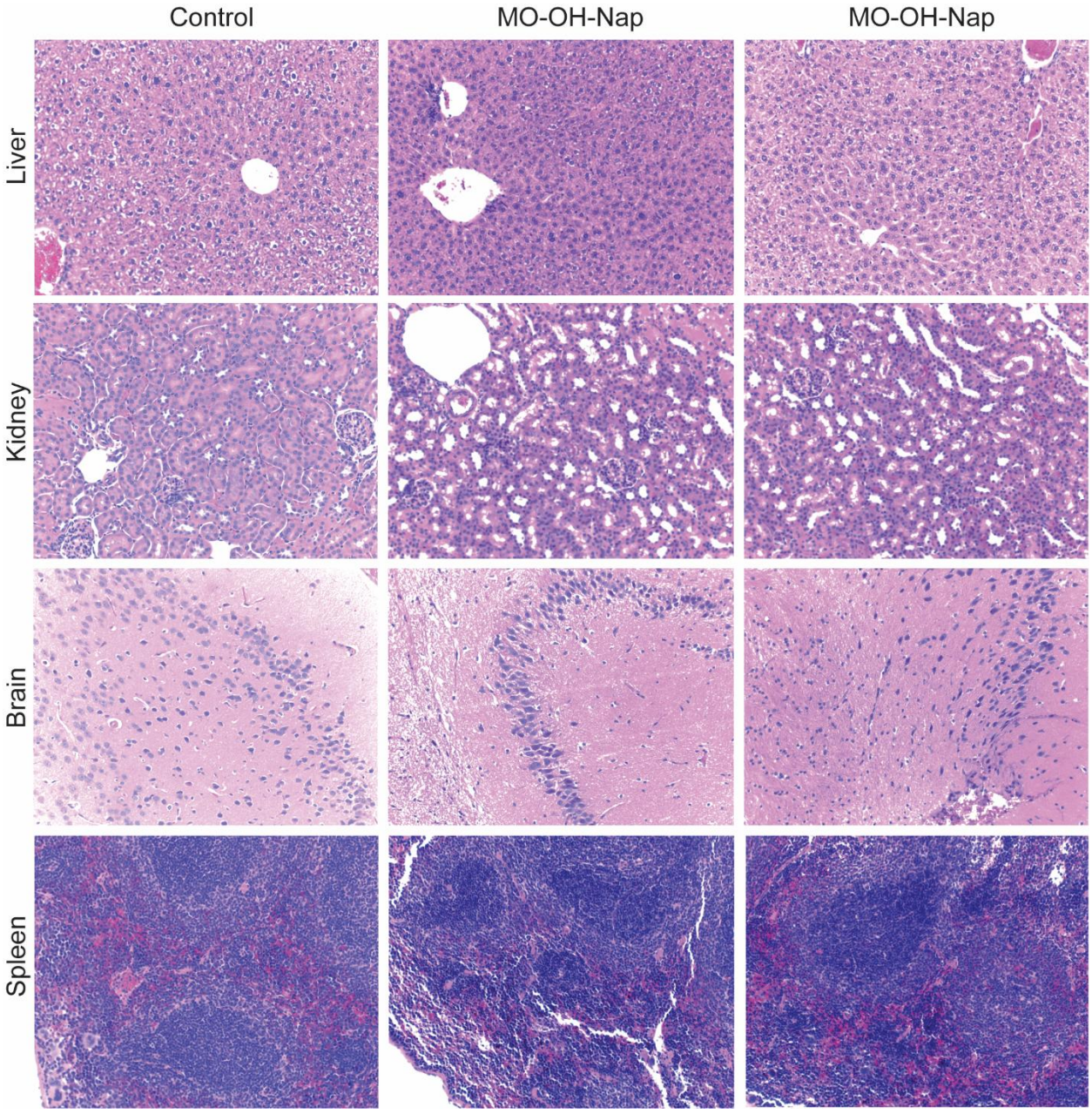
Supplmental Figure 6. MOOH induces cleavage of PERK. RPMI-8226 and MM.1S cells were treated for 48 hours with DMSO, MO-OH-Nap or DFO in the presence or absence of FC and AFS. Protein levels of PERK and β-tubulin were measured by immunoblot. Immunoblots are representative of three independent experiments.



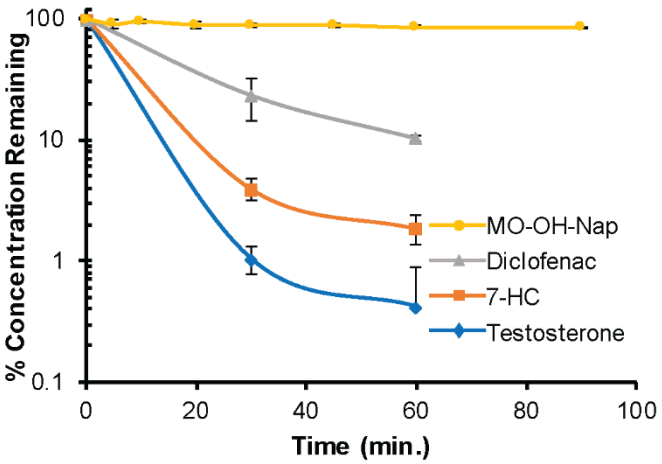
Supplemental Figure 7. DFO and MO-OH-Nap induce upregulation of transferrin receptor protein. a) Flow cytometry analysis of TFR1 surface protein. Flow histograms are representative examples where the x-axis represents TFR1 levels. b) and c) Immunoblot of TFR1 protein levels in U266 cells following 48-hour treatment with (b) DMSO (control), DFO, or MO-OH-Nap (MOOH) and (c) MOOH in combination with 50 μM $FeCl_3$ (FC) or $FeSO_4$ (AFS). β-tubulin is shown as a loading control. Relative fold change values for TFR1 are shown (calculated using densitometry in ImageJ and normalized to loading control).



Supplemental Figure 8. MO-OH-Nap does not induce changes in reactive oxygen species in MM cells. a) Summary of flow cytometry analysis of H2DCFDA fluorescence in U266, RPMI-8226, and MM.1S cells treated with MO-OH-Nap (denoted as MOOH; 2.5 μM for RPMI-8226/MM.1S and 10 μM U266) or 40 μM DFO with or without 50 μM FC. Data are shown as mean ± S.D. (n=3). b) Representative flow examples of 24 and 48 hour MO-OH-Nap treatment. Fluorescence of CM-H2DCFDAis shown on the X-axis.



Supplemental Figure 9. Repeat dosing of MO-OH-Nap does not cause changes in organ histology. H&E sections of liver, spleen, kidney, and brain of CD-1 mice injected with 9.4mg/kg MO-OH-Nap three times per week for four weeks.



Supplemental Figure 10. MO-OH-Nap is metabolically stable Time-dependent metabolic depletion (% turnover or amount remaining vs. incubation time) of MO-OH-Nap, testosterone, 7-HC (7 hydroxycoumarin) and diclofenac in mouse S9 fraction. Data shown as mean ± S.D (n=3).

Antibody	Company	Cat. #	Dilution
ATF-4	Cell Signaling	11815	1:1000
CHOP	Cell Signaling	2895	1:300
Cleaved Caspase 3	Cell Signaling	9664	1:500
Cleaved Caspase 8	Cell Signaling	9496	1:500
Cleaved Caspase 9	Cell Signaling	9501	1:500
eIF2α	Cell Signaling	9722	1:1000
p-eIF2α	Cell Signaling	3597	1:1000
IRE1α	Cell Signaling	3294	1:2000
PERK	Santa Cruz	sc-377400	1:1000
PARP	Santa Cruz	sc-7150	1:10,000
β-Tubulin	Sigma	T5201	1:25,000
TFR1	Santa Cruz	sc-32272	1:250
Acetyl-Histone H3 (Lys 9)	Cell Signaling	9649	1:3000
Acetyl-Histone H3 (Lys 23)	Cell Signaling	14932	1:1000

Supplementary Table 1: Antibodies used for western blotting.

	Normal	9.4mg/kg 3x/wk
WBC	3-15 10^3/mm^3	5.65
RBC	5-12 10^6/mm^3	8.445
HGB	11-18 g/dl	15.15
HCT	36-52%	42.75
PLT	140-600 10^3/mm^3	899.5
MCV	44 -69 fl	51
MCH	12 -24.5 pg	18
MCHC	21.6-42 g/dl	35.35
RDW	21 -25%	12.45
MPV	4.6 -7.3 fl	5.3
% LYMP	17 -48%	55.75
%MON	4 -10%	11.55
%GRA	43 -76%	32.7
#LYM	1.2-3.2 10^3/mm^3	3.1
#MOH	0.3-0.8 10^3/mm^3	0.6
#GRA	1.2-6.8 10^3/mm^3	1.95

Supplemental Table 2. CBC analysis. Mice were injected IV with 9.4 mg/kg MO-OH-Nap 3 times per week for four weeks (n=2). Blood was collected at the time of harvest. Abbreviation: white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin count (MCHC), red cell distribution (RDW), mean platelet volume (MPV), lymphocytes (LYM), monocytes (MON), and granulocytes (GRA)

	Normal	9.4mg/kg 3x/wk
BUN (MG/DL)	9-33	17.5
CRE (MG/DL)	0.2-0.9	0.2
ALT (U/L)	17-73	38.5
ALP (U/L)	35-222	57.5
AST (U/L)	54-298	162
TBIL (MG/DL)	0-0.9	0.3
GLU (MG/DL)	140-263	233
CA (MG/DL)	6-13	11.15
TP (G/DL)	3.9-6.4	5.8
ALB (G/DL)	2.5-4.6	4.45
GLOB (G/DL)	1.2-2.2	0.85
Na (mmol/L)	110-195	150.5
K (mmol/L)	4-10.5	8.5
Cl^- (mmol/L)	110-128	110
TCO2 (mmol/L)	NR	27

Supplemental Table 3. Blood chemistry panel. Mice were injected IV with 9.4 mg/kg MO-OH-Nap 3 times per week for four weeks (n=2). Blood was collected at the time of harvest. Abbreviations: blood urea nitrogen (BUN), creatinine (CRE), alanine amino-transferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin (TBIL), glucose (GLU), total protein (TP), albumin (ALB), globulin (GLOB) and NR (not reported)