Electronic Supplementary Information

Multicellular aggregation of maltol-modified cells triggered by Fe^{3+} ions

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Supplementary Data and Images

General Experimental

Chemicals, solvents and reagents used are commercially available and were used without further purification. PE refers to petroleum ether, bp 40-60 °C. TLCs were carried out on Merck Aluminium backed TLC plates Silica Gel 60 F254 and viewed using UV light of wavelength 254 nm and then stained with potassium permanganate. Merck Silica Gel (0.040-0.063 mm) was used for column chromatography. Compounds were loaded as an oil, CH_2Cl_2 solution or dry loaded by adsorption onto silica. Melting points were obtained using a Reichert-Jung heated-stage microscope. Infrared spectra were recorded on a Perkin-Elmer Spectrum RXI FT-IR system and all values are recorded in cm^{-1}.

NMR spectra were obtained on a Bruker Avance III (500 MHz) spectrometer. The chemical shifts are recorded in parts per million (ppm) with reference to tetramethylsilane. The coupling constants *J* are quoted to the nearest 0.5 Hz and are not corrected. The multiplicities are assigned as a singlet (s), doublet (d), triplet (t), doublet of doublets (dd), quartet (q) and multiplet (m). Mass spectra and high resolution mass spectra were obtained on a micrOTOF™ from Bruker Daltonics (Bremen, Germany) coupled with an electrospray source (ESI-TOF) using an autosampler in an Agilent 1100 LC system. Data was processed using external calibration with the Bruker Daltonics software, DataAnalysis™ as part of the overall hardware control software, Compass 1.1™.

All images were taken on a Leica DMI4000B inverted microscope. Optical density (OD) readings were obtained on a BMG Labtech Fluostar plate reader with NUNC six-well flat bottom plates at room temperature. Data processing and analysis was performed using Microsoft Excel.

Preparation of Agarose-Coated Plates[1]

Plates were coated with agarose to prevent cell adhesion during aggregation experiments. The agarose-coated plates were prepared as previously described.[1]

Materials

1) Agarose (molecular biology grade)
2) Tissue culture medium
3) Tissue culture-treated six-well plates

Preparation

1) 0.9% w/v agarose stock was prepared from 0.9 g agarose in 100 mL of growth-factor free medium.
2) The suspension was boiled in a microwave oven to dissolve the agarose and sterilise the solution.
3) The solution was pipetted into the plates in a biosafety cabinet. Approximately 2 mL was used per well of a six-well plate.
4) Plates were left open in the biosafety cabinet at room temperature, until solidified and used either directly or stored and used later.
5) For storage, a small amount of medium was added to each well (approx. 2 mL) to prevent drying out, sealed with a plastic wrap and stored at 4 °C.

MTS Cell Proliferation Assay[2]

Human cancer cell lines HT29 and MDA-MB-231 were supplied by Cancer Research UK. They were maintained in DMEM with high glucose (4.5 g/L) and 2mM L-glutamine, supplemented with penicillin 100 U/mL, streptomycin 100 μg/mL and foetal bovine serum at 10%. All reagents were supplied by Invitrogen.

The MTS assays of the maltol-hydrazide **6** followed the previously reported procedure,[3] and were performed twice with HT29 and once with MDA-MB-231.

A modified MTS protocol, outlined below, was used to determine cell proliferation in the six-well plates of unmodified and maltol-modified HT29 cells in the presence and absence of 50 μM Fe^{3+} after 24, 48 and 72 h incubation.

1. Cells were maintained in 75 cm^2 tissue culture flasks (Nunc) with a weekly 1:10 split.

2. Three sets of six-well plates were prepared following the procedure outlined in "Cell Aggregation" (next section). After 24 h of agitation, the first set of plates were treated with MTS.

3. 200 μL of MTS reagent was added to each well of a six-well plate containing unmodified and modified HT29 cells with and without 50 μM Fe^{3+}.

4. Plates were incubated at 37 °C, in humidified 5% CO_2 in air for 2 h.

5. Optical density readings were taken at 490 nm after 2 h.

6. Means and standard errors were calculated from background corrected OD_{490nm} values.

7. The same steps were taken after 48 h with the second set of six-well plates and again after 72 h with the third set of plates. The whole process was performed in triplicate and the results shown in **Fig. 3**.

Note: This assay is based upon the development of a coloured metabolite from viable cells. Therefore the inhibition of colour development by an active agent does not distinguish between inhibition of cell metabolism *ie* cytostasis and reduction in cell number *ie* cytotoxicity. Nevertheless, this assay provides a very quick and easy first approach for screening test compounds.

Cell Aggregation

Cell aggregation was performed following the procedures previously reported,[4] but using the maltol-derived hydrazide **6** and HT29 (and MDA-MB-231) cells.

1. Cells were grown to confluence, suspended by brief trypsinisation and washed with room temperature phosphate-buffered saline (PBS). Cells were then resuspended in sodium periodate solution (1 mM in PBS) at 4 °C, incubated in the dark at 4 °C for 10 mins, diluted with PBS and pelleted.

2. The cells were incubated in 10 mL maltol hydrazide **6** solution (100 μM in PBS) for 60 min at room temperature with constant agitation (maximum setting).

3. The cells were then diluted with PBS and pelleted, resuspended in serum free culture medium (DMEM), counted and a stock suspension of 1.0 x 10^6 cells / mL obtained.

4. 1 mL of this cell suspension was plated into each well of an agarose-coated six-well plate followed by 1 mL of an 100 μM $FeCl_3$ solution in PBS, giving a final concentration of 5 x 10^5 cells / mL in 50 μM $FeCl_3$.

5. The plate was then left at room temperature with constant agitation (maximum setting) for various times and images taken on a Leica DMI400B inverted microscope.

6. The same method was applied for the heteroaggregates with the additional step of fluorescently labelling the HT29 cells green and MDA-MB-231 red using CellTracker™ (Invitrogen) prior to $NaIO_4$ oxidation according to the manufacturer's instructions.

Optimisation Experiments

Cell aggregation was performed following the procedures previously reported,[4] but using the maltol-derived hydrazide **6** and HT29 cells and optimised using the following procedures.

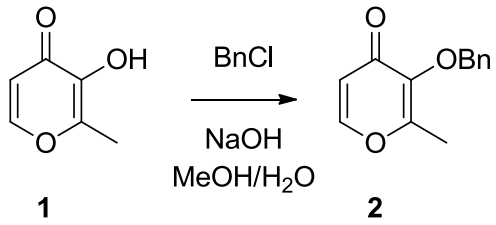
1. Cells were prepared and plated as previously described except with either varying concentrations of Fe^{3+} or different agitation times in agarose-coated six-well plates with a final volume of 2 mL.

2. Three randomly selected images were taken at each time-point and the apparent aggregate area calculated using the area function in ImageJ,[5] which was calibrated in micrometers using the microscope image dimensions.

3. The average apparent aggregate area ± standard error of each time-point from three independent experiments was then calculated and plotted using Microsoft Excel and the data shown in **Fig. 2**.

Experimental

3-(Benzyloxy)-2-methyl-4*H*-pyran-4-one (2)[6,7]

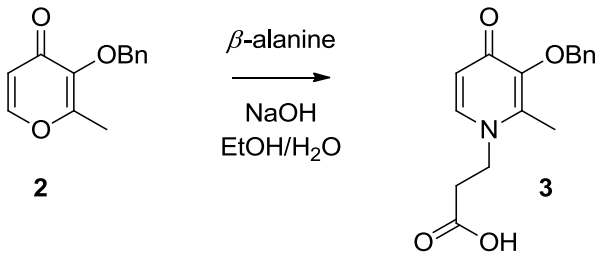


Following the procedure previously reported,[6] sodium hydroxide (6 g, 0.15 mol) in water (20 mL) was added to a stirred solution of maltol **1** (17.8 g, 0.14 mol) in MeOH (180 mL), followed by benzyl chloride (20.9 g, 0.16 mol) and the mixture was heated to reflux for 12 h. The solvent was partially removed under reduced pressure to afford an orange oil which was dissolved in CH_2Cl_2 (80 mL) and washed with 5% (w/v) aqueous sodium hydroxide (5 x 30 mL) and water (2 x 50 mL). The organic fraction was dried over anhydrous sodium sulfate and filtered. The solvent was removed by rotary evaporation to give the benzylated product **2** as a pale yellow oil (26.3 g, 87%).

R_f [PE-EtOAc 4:6] 0.65; **IR** ν_{max}(film)/cm^{-1} 1633, 1431 and 1173; **^{1}H NMR** δ_H (500 MHz; $CDCl_3$); 2.09 (3 H, s, CH_3), 5.16 (2 H, s, Bn CH_2), 6.36 (1 H, d, *J* 5.0 Hz, COC*H*=CH), 7.33-7.39 (5 H, m, Ph CH) and 7.59 (1 H, d, *J* 5.0, COCH=C*H*); **^{13}C NMR** δ_C (125 MHz; $CDCl_3$); 14.8 (CH_3), 73.6 (CH_2), 117.2 (CH), 128.3 (CH), 128.4 (CH), 129.0 (CH), 136.9 (Cq), 143.8 (Cq), 153.4 (Cq), 159.7 (CH) and 175.1 (Cq); **MS** m/z (ES^+) Found 217.0861 (MH^+), $C_{13}H_{13}O_3$ (MH^+) requires 217.0865.

Consistent with the ^{1}H NMR data previously reported for this compound.[8]

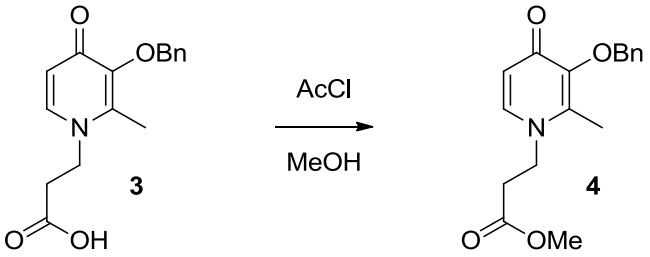
3-(3-(Benzyloxy)-2-methyl-4-oxopyridin-1(4*H*)-yl)propanoic acid (3)[6,7]



Following the procedure previously reported,[6] except using twice the amount of *β*-alanine and using a different work-up, *β*-alanine (8.7 g, 97.8 mmol) was added to a stirred solution of benzylated maltol **1** (8.5 g, 39.4 mmol) in EtOH (100 mL) and water (100 mL), followed by 10 M sodium hydroxide solution until pH 13 was achieved. After heating at reflux for 18 h, the solvent was partially removed under reduced pressure and water was added followed by hydrochloric acid to adjust to pH 4. The yellow precipitate was filtered and dried to afford the acid **3** as a pale yellow solid (7.9 g, 70%).

R_f [CH_2Cl_2-MeOH 9:1] 0.21; **Mp** 172-173 °C (EtOH), (Lit.[6] 170-171 °C); **IR** ν_{max}(film)/cm^{-1} 1729, 1625 and 1550; **^{1}H NMR** δ_H (500 MHz; DMSO); 2.21 (3 H, s, CH_3), 2.66 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 4.11 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 5.00 (2 H, s, Bn CH_2), 6.20 (1 H, d, *J* 7.5 Hz, COC*H*=CH), 7.32-7.41 (5 H, m, Ph CH) and 7.66 (1 H, d, *J* 7.5 Hz, COCH=C*H*); **^{13}C NMR** δ_C (125 MHz; DMSO); 12.0 (CH_3), 34.5 (CH_2), 48.6 (CH_2), 72.0 (CH_2), 115.9 (CH), 122.1 (Cq), 127.9 (CH), 128.3 (CH), 128.4 (CH), 137.7 (Cq), 139.8 (CH), 145.0 (Cq), 171.9 (Cq) and 172.0 (Cq); **MS** m/z (ES^+) Found 288.1238 (MH^+) and 310.1055 (MNa^+), $C_{16}H_{18}N_1O_4$ (MH^+) requires 288.1236 and $C_{16}H_{17}N_1NaO_4$ (MNa^+) requires 310.1055.

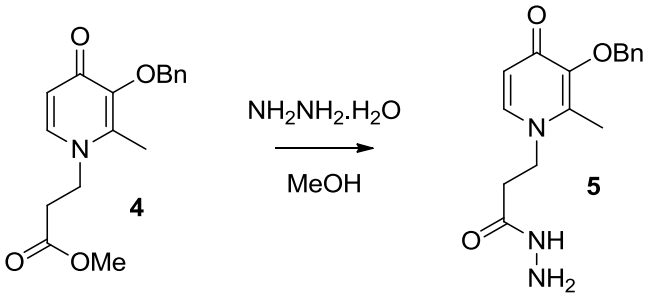
Methyl 3-(3-(benzyloxy)-2-methyl-4-oxopyridin-1(4*H*)-yl)propanoate (4)[7]



Acetyl chloride (0.23 g, 3.0 mmol) was added dropwise to a stirred solution of MeOH (5.0 mL) on ice, the solution was then allowed to warm to rt. The acid **3** (0.57 g, 2.0 mmol) was dissolved in MeOH (5 mL) and added dropwise to the solution and the reaction heated to reflux for 1 h. After 1 h the solvent was removed under reduced pressure to afford, after recrystallisation from MeOH, the methyl ester **4** as a white solid (0.59 g, 98%).

R_f [CH_2Cl_2-MeOH 9:1] 0.61; **Mp** 144-145 °C (MeOH); **IR** ν_{max}(film)/cm^{-1} 1742, 1633, 1186; **^{1}H NMR** δ_H (500 MHz; $CDCl_3$); 2.09 (3 H, s, CH_3), 2.63 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 3.70 (3 H, s, OCH_3), 4.08 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 5.21 (2 H, s, Bn CH_2), 6.42 (1 H, d, *J* 7.5 Hz, COC*H*=CH) and 7.27-7.40 (6 H, m, Ph CH and COCH=C*H*); **^{13}C NMR** δ_C (125 MHz; $CDCl_3$); 12.4 (CH_3), 34.8 (CH_2), 48.9 (CH_2), 52.3 (OCH_3), 73.0 (CH_2), 117.5 (CH), 128.0 (CH), 128.2 (CH), 129.1 (CH), 137.5 (Cq), 138.4 (CH), 140.2 (Cq), 146.2 (Cq), 170.3 (Cq) and 173.4 (Cq); **MS** m/z (ES^+) Found 324.1201 (MNa^+), $C_{17}H_{19}N_1NaO_4$ (MNa^+) requires 324.1212.

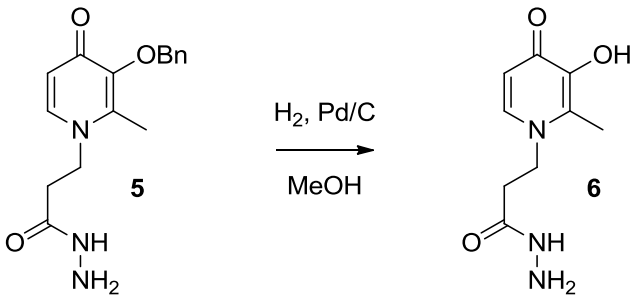
3-(3-(Benzyloxy)-2-methyl-4-oxopyridin-1(4*H*)-yl)propanehydrazide (5)



Hydrazine monohydrate (0.64 g, 12.8 mmol) was added to a stirred solution of the methyl ester **4** (1.5 g, 5.0 mmol) in MeOH (10 mL) at room temperature and heated at reflux for 18 h. After 18 h the solvent was removed under reduced pressure and the residue purified by column chromatography with silica gel using CH_2Cl_2:MeOH 2:8 solvent system to afford the protected hydrazide product **5** as a pale yellow solid (0.93 g, 62%).

R_f [CH_2Cl_2-MeOH 9:1] 0.09; **M.p** 76-78 °C (MeOH); **IR** ν_{max}(film)/cm^{-1} 1664, 1629 and 1127; **^{1}H NMR** δ_H (400 MHz; DMSO); 2.27 (3 H, s, CH_3), 2.48 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 4.15 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 4.38 (2 H, br s, NH_2), 5.07 (2 H, s, Bn CH_2), 6.18 (1 H, d, *J* 7.5 Hz, COC*H*=CH), 7.37-7.48 (5 H, m, Ph CH), 7.57 (1 H, d, *J* 7.5 Hz, COCH=C*H*) and 9.16 (1 H, s, NH); **^{13}C NMR** δ_C (125 MHz; DMSO); 11.9 (CH_3), 34.3 (CH_2), 49.2 (CH_2), 71.9 (CH_2), 116.0 (CH), 116.1 (CH), 127.9 (CH), 128.3 (CH), 137.8 (Cq), 139.6 (CH), 140.8 (Cq), 145.3 (Cq), 168.2 (Cq) and 171.8 (Cq); **MS** m/z (ES^+) Found 302.1480 (MH^+) and 324.1310 (MNa^+), $C_{16}H_{20}N_3O_3$ (MH^+) requires 302.1505 and $C_{16}H_{19}N_3NaO_3$ (MNa^+) requires 324.1324.

3-(3-Hydroxy-2-methyl-4-oxopyridin-1(4*H*)-yl)propanehydrazide (6)



The benzyl protected maltol hydrazide **5** (0.93 g, 3.1 mmol) was added to a stirred solution of 10% w/w Pd/C (0.62 mg, 20 mol%) in MeOH (10 mL). Air was purged and the reaction placed under a balloon of H_2 and stirring continued at room temperature for 24 h. The solution was filtered through filter paper and cotton wool and the solvent removed under reduced pressure to give, after recrystallisation from MeOH, the maltol hydrazide **6** as a beige coloured solid (0.55 g, 84%).

R_f [CH_2Cl_2-MeOH 9:1] 0.06; **Mp** 102-104^{o}C (MeOH); **IR** ν_{max}(film)/cm^{-1} 3616, 1668 and 1626; **^{1}H NMR** δ_H (500 MHz; DMSO); 2.35 (3H, s, CH_3), 2.56 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 4.26 (2 H, t, *J* 7.0 Hz, NCH_2CH_2), 5.50-7.00 (3H, br s, NH and NH_2), 6.38 (1 H, d, *J* 7.0 Hz, COC*H*=CH), 7.65 (1 H, d, *J* 7.0 Hz, COCH=C*H*) and 9.71 (1 H, br s, OH); **^{13}C NMR** δ_c (125 MHz; DMSO); 11.6 (CH_3), 34.1 (CH_2), 49.8 (CH_2), 110.7 (CH), 131.8 (Cq), 137.9 (CH), 144.8 (Cq), 166.6 (Cq) and 168.2 (Cq); **MS** m/z (ES$^+$) Found 212.1033 (MH$^+$) and 234.0838 (MNa$^+$), $C_9H_{14}N_3O_3$ (MH$^+$) requires 212.1035 and $C_9H_{13}N_3NaO_3$ (MNa$^+$) requires 234.0855.

Acknowledgements

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References

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5. ImageJ available from http://rsb.info.nih.gov/ij/.

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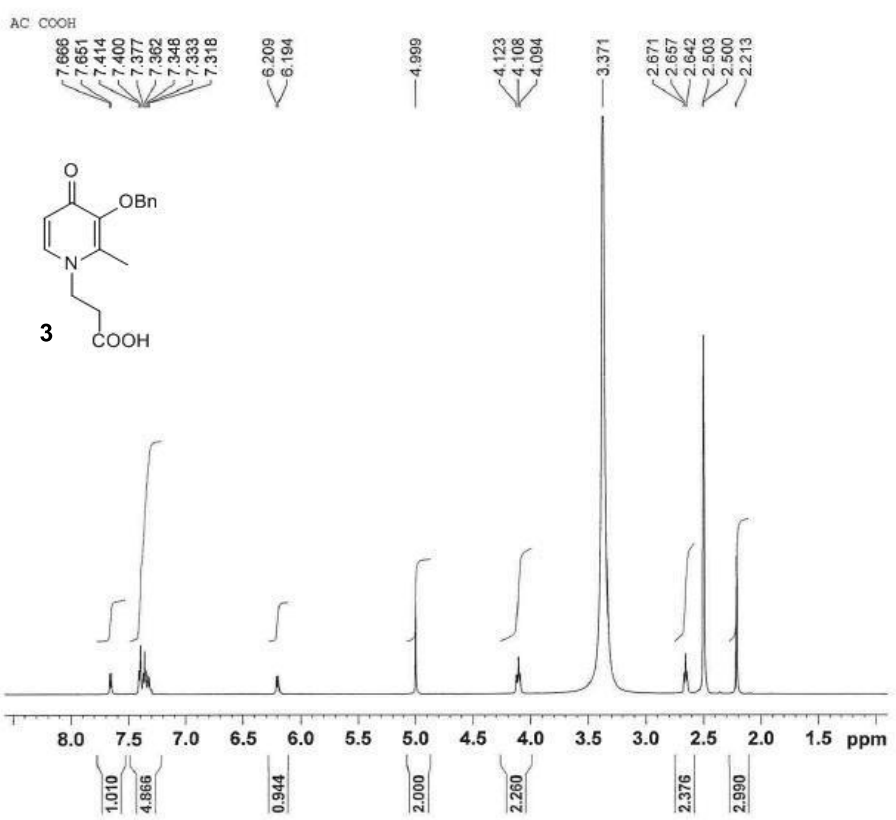
Supplementary Data and Images

^{1}H and ^{13}C NMR Spectra

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FIDRES             0.157632 Hz
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RG                       181
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TE                    294.5 K
D1                 1.00000000 sec
TD0                        1

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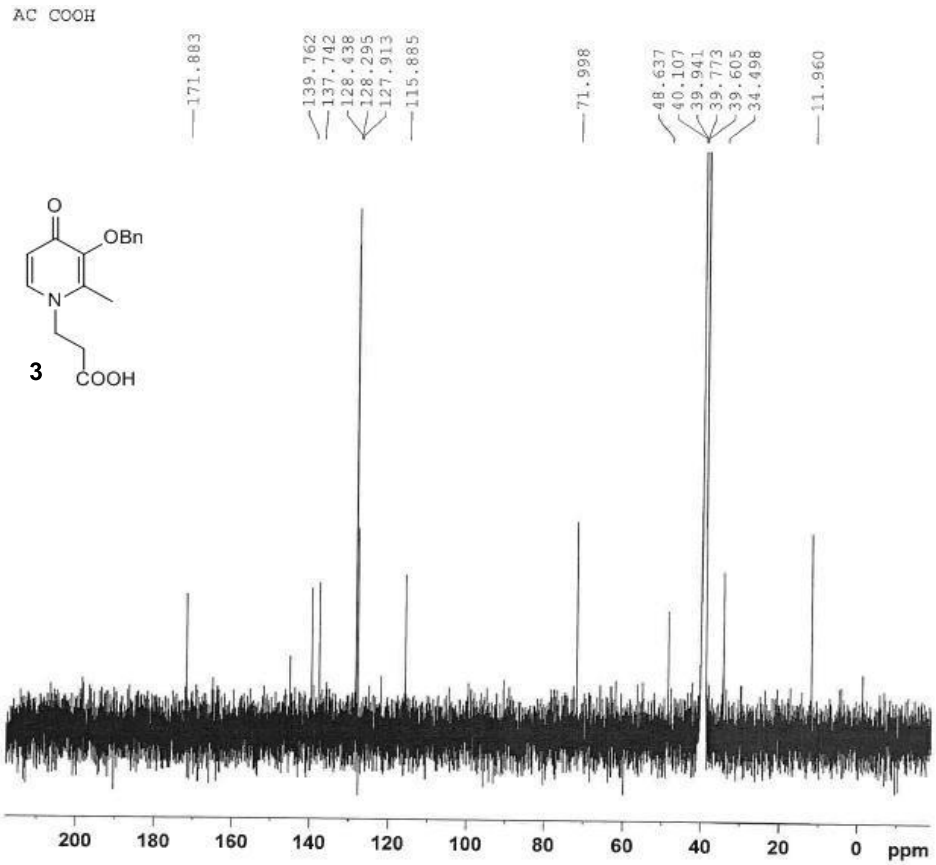


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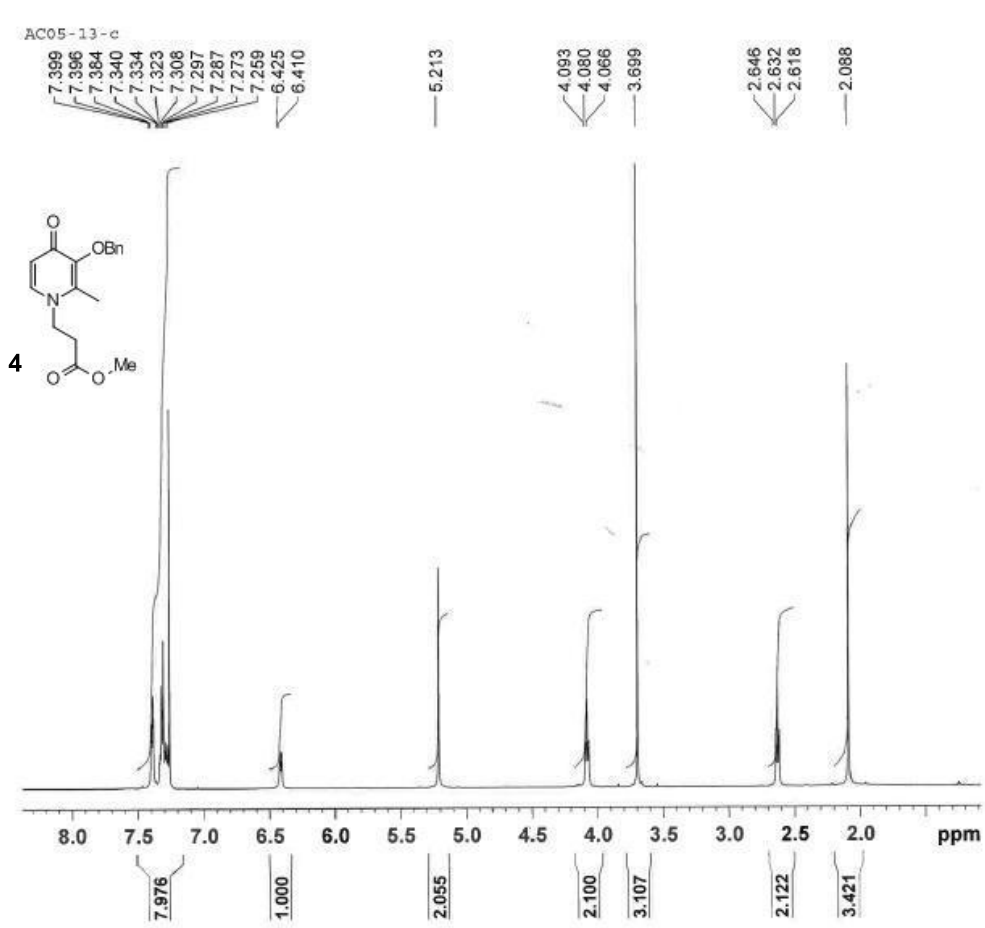


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TD0                         1

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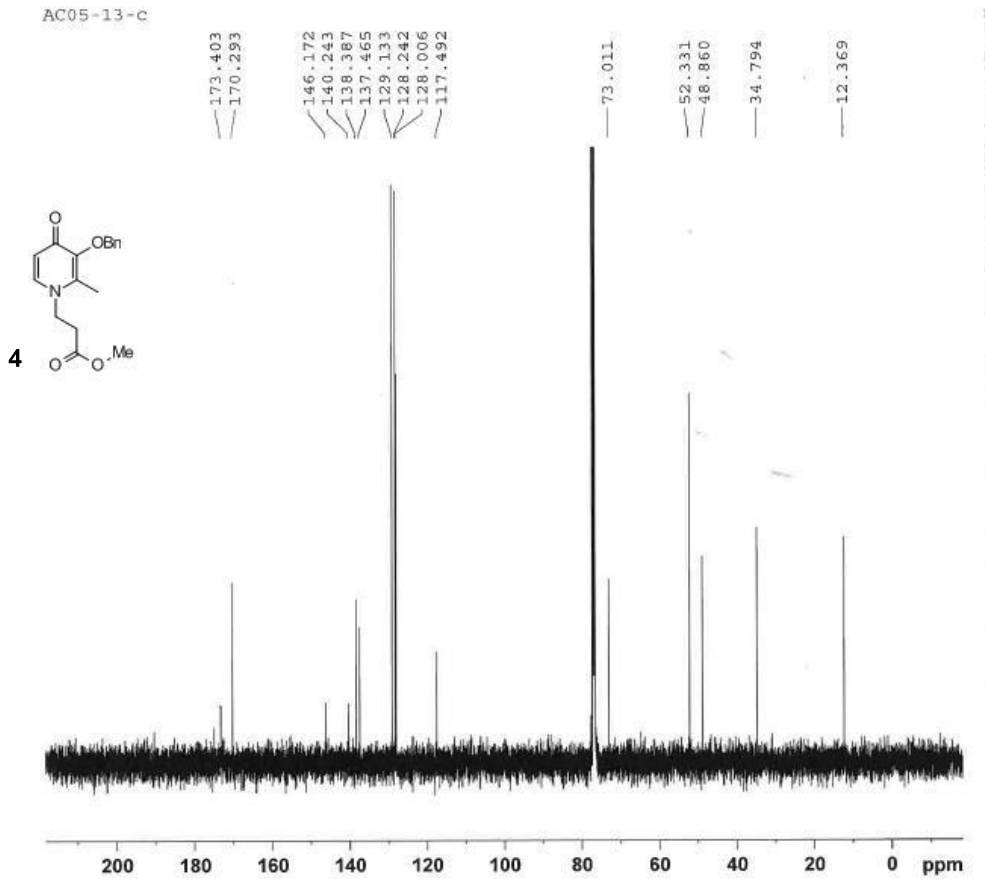


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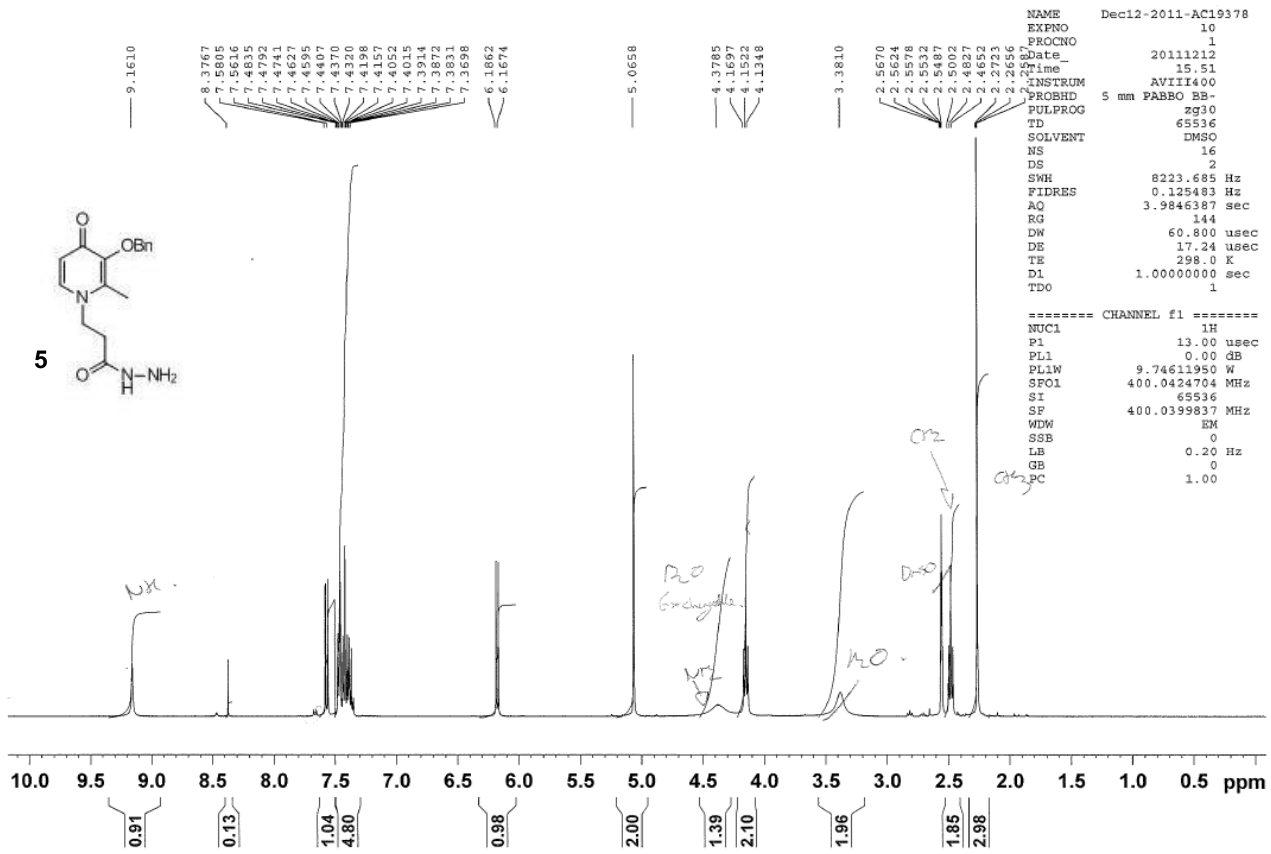
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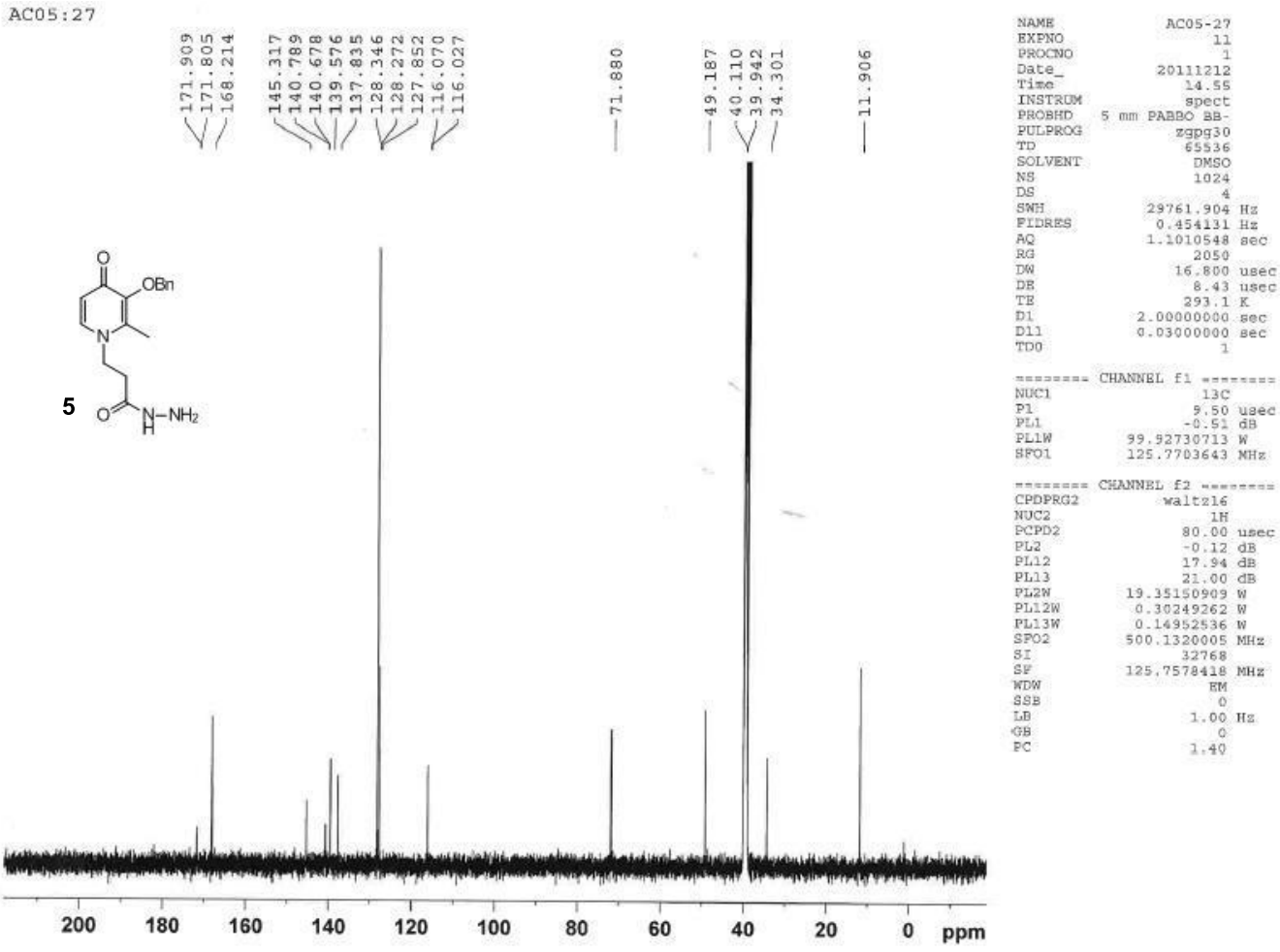
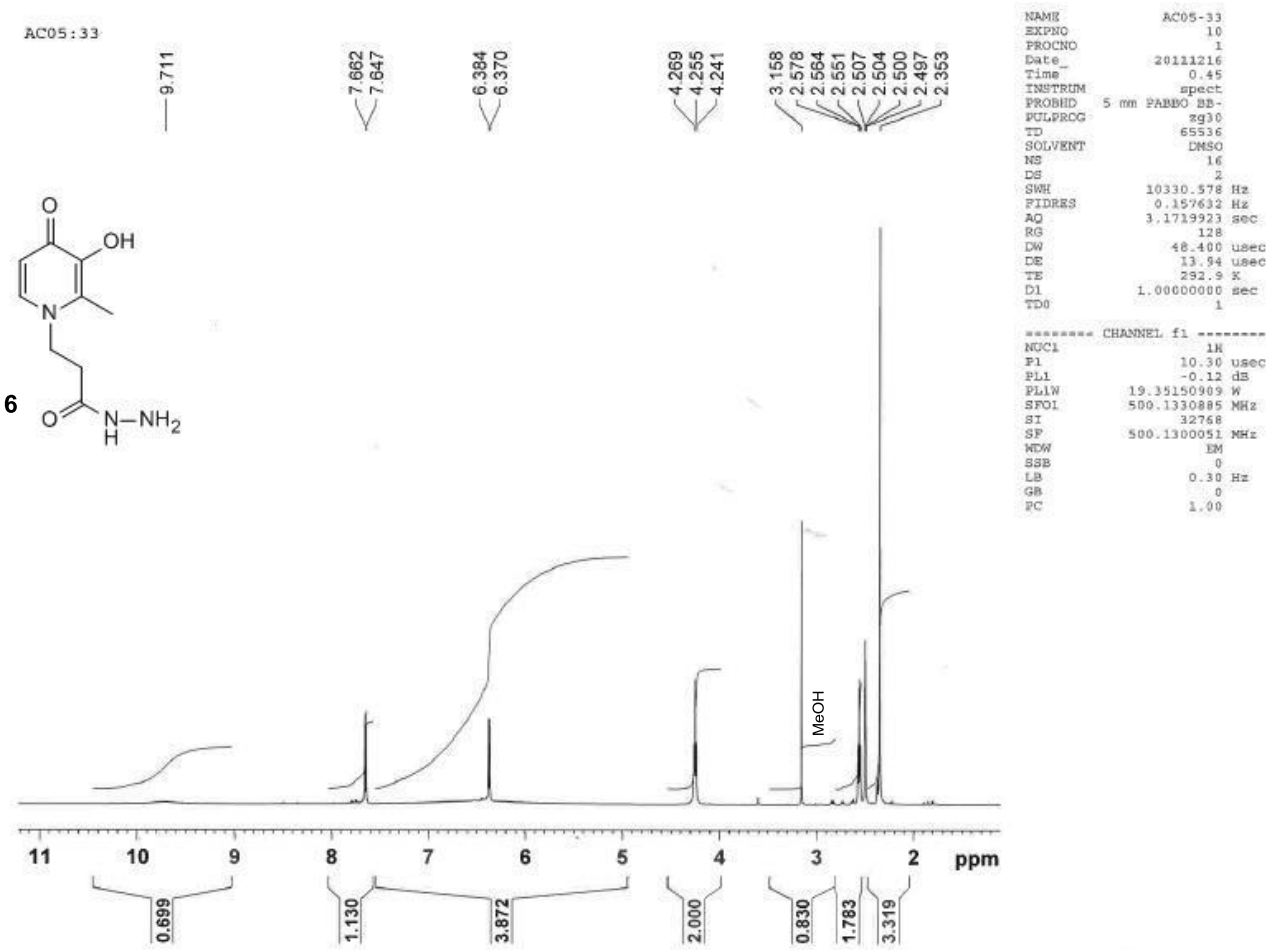
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 16
DS 2
SWH 8223.685 Hz
FIDRES 0.125483 Hz
AQ 3.9846387 sec
RG 144
DW 60.800 usec
DE 17.24 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 13.00 usec
PL1 0.00 dB
PL1W 9.74611950 W
SFO1 400.0424704 MHz
SI 65536
SF 400.0399837 MHz
WDW EM
SSB 0
LB 0.20 Hz
GB 0
PC 1.00
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
0.91 0.13 1.04 4.80 0.98 2.00 1.39 2.10 1.96 1.85 2.98


AC05:27
171.909
171.805
168.214
145.317
140.789
140.678
139.576
137.835
128.346
128.272
127.852
116.070
116.027
71.880
49.187
40.110
39.942
34.301
11.906
OBn
5
N–NH2
NAME AC05-27
EXPNO 11
PROCNO 1
Date_ 20111212
Time 14.55
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 1024
DS 4
SWH 29761.904 Hz
FIDRES 0.454131 Hz
AQ 1.1010548 sec
RG 2050
DW 16.800 usec
DE 8.43 usec
TE 293.1 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 9.50 usec
PL1 -0.51 dB
PL1W 99.92730713 W
SFO1 125.7703643 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -0.12 dB
PL12 17.94 dB
PL13 21.00 dB
PL2W 19.35150909 W
PL12W 0.30249262 W
PL13W 0.14952536 W
SFO2 500.1320005 MHz
SI 32768
SF 125.7578418 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
200 180 160 140 120 100 80 60 40 20 0 ppm

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NAME                 AC05-33
EXPNO                     10
PROCNO                     1
Date_               20111216
Time                    0.45
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG                zg30
TD                    65536
SOLVENT                DMSO
NS                        16
DS                         2
SWH              10330.578 Hz
FIDRES           0.157632 Hz
AQ               3.1719923 sec
RG                       128
DW                  48.400 usec
DE                  13.94 usec
TE                   292.9 K
D1               1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
NUC1                      1H
P1                  10.30 usec
PL1                  -0.12 dB
PL1W            19.35150909 W
SFO1            500.1330885 MHz
SI                      32768
SF              500.1300051 MHz
WDW                        EM
SSB                         0
LB                   0.30 Hz
GB                          0
PC                       1.00
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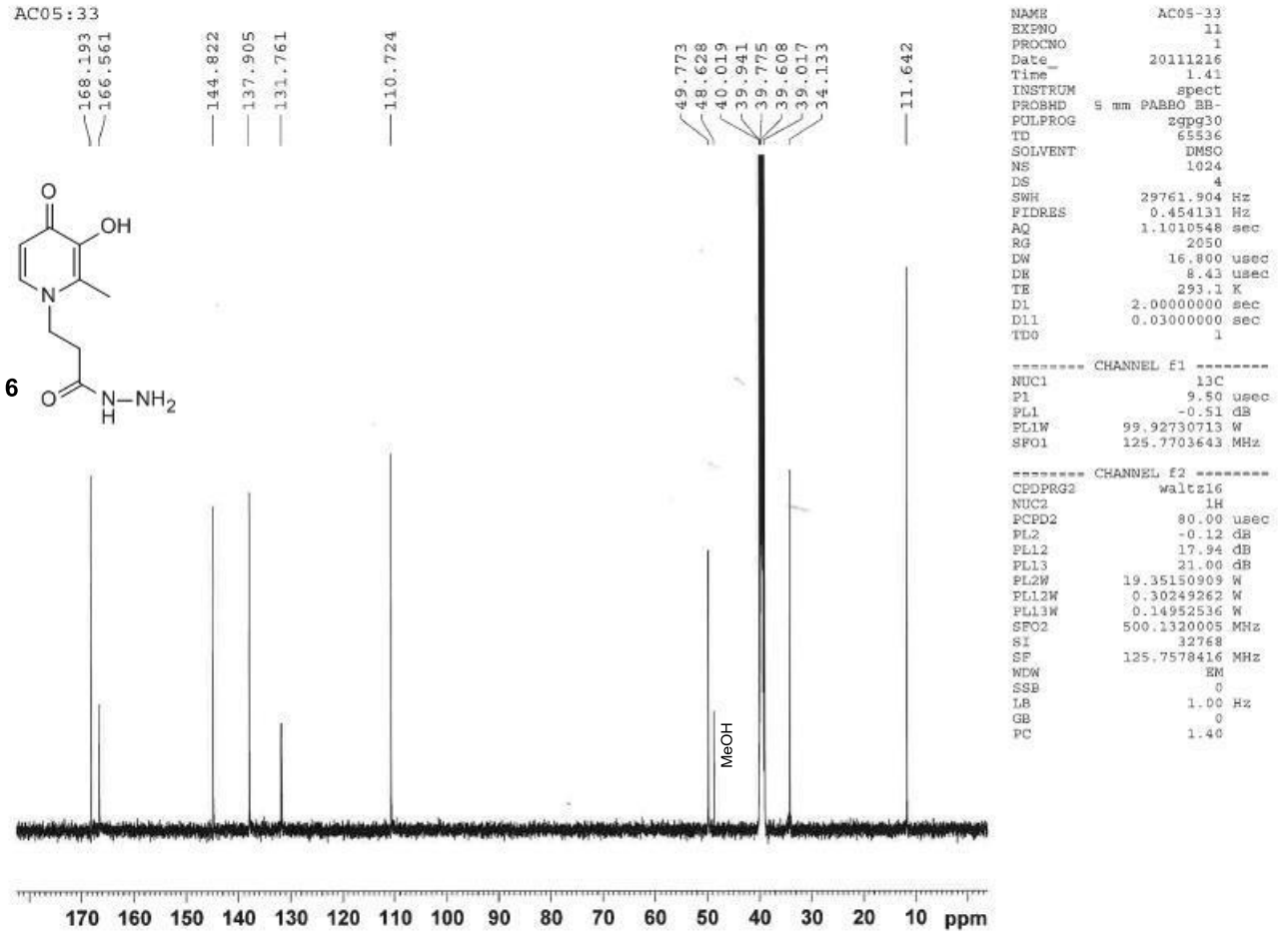


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NAME                 AC05-33
EXPNO                     11
PROCNO                     1
Date_               20111216
Time                    1.41
INSTRUM                spect
PROBHD   5 mm PABBO BB-
PULPROG              zgpg30
TD                    65536
SOLVENT                DMSO
NS                      1024
DS                         4
SWH              29761.904 Hz
FIDRES           0.454131 Hz
AQ               1.1010548 sec
RG                      2050
DW                  16.800 usec
DE                   8.43 usec
TE                   293.1 K
D1               2.00000000 sec
D11              0.03000000 sec
TD0                        1

======== CHANNEL f1 ========
NUC1                     13C
P1                   9.50 usec
PL1                  -0.51 dB
PL1W            99.92730713 W
SFO1            125.7703643 MHz

======== CHANNEL f2 ========
CPDPRG2              waltz16
NUC2                      1H
PCPD2               80.00 usec
PL2                  -0.12 dB
PL12                 17.94 dB
PL13                 21.00 dB
PL2W            19.35150909 W
PL12W            0.30249262 W
PL13W            0.14952536 W
SFO2            500.1320005 MHz
SI                      32768
SF              125.7578416 MHz
WDW                        EM
SSB                         0
LB                   1.00 Hz
GB                          0
PC                       1.40
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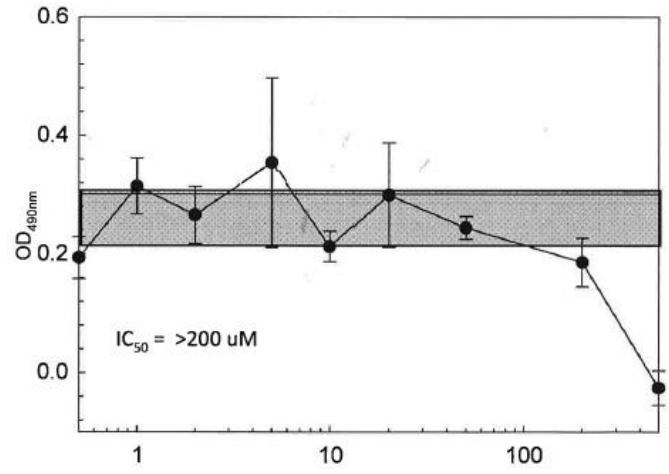
MTS Assays – Hydrazide 6

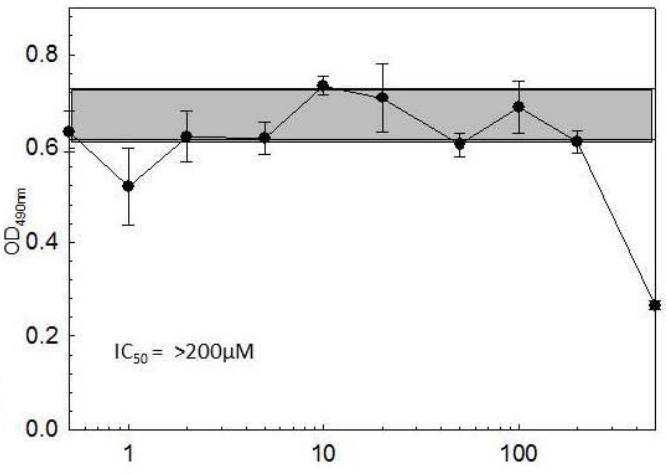
HT29 Human Colon Carcinoma
3 Day Exposure MTS
Hydrazide 6



Hydrazide **6** Concentration (μM)

1% DMSO only
Points are means ± s.d
n = 4

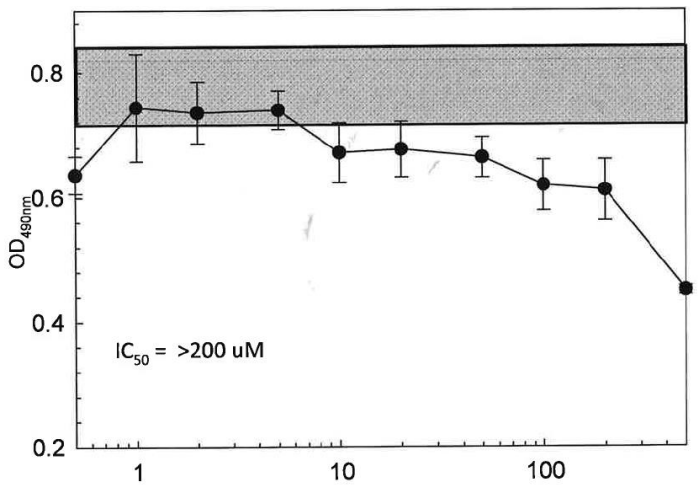
HT29 Human Colon Carcinoma
3 Day Exposure MTS
Hydrazide 6



Hydrazide **6** Concentration (μM)

1% DMSO only
Points are means ± s.d
n = 4

MDA231 Human Breast Carcinoma
3 Day Exposure MTS
Hydrazide 6



Hydrazide **6** Concentration (μM)

1% DMSO only
Points are means ± s.d
n = 4

HT29 cell Aggregation Experiments

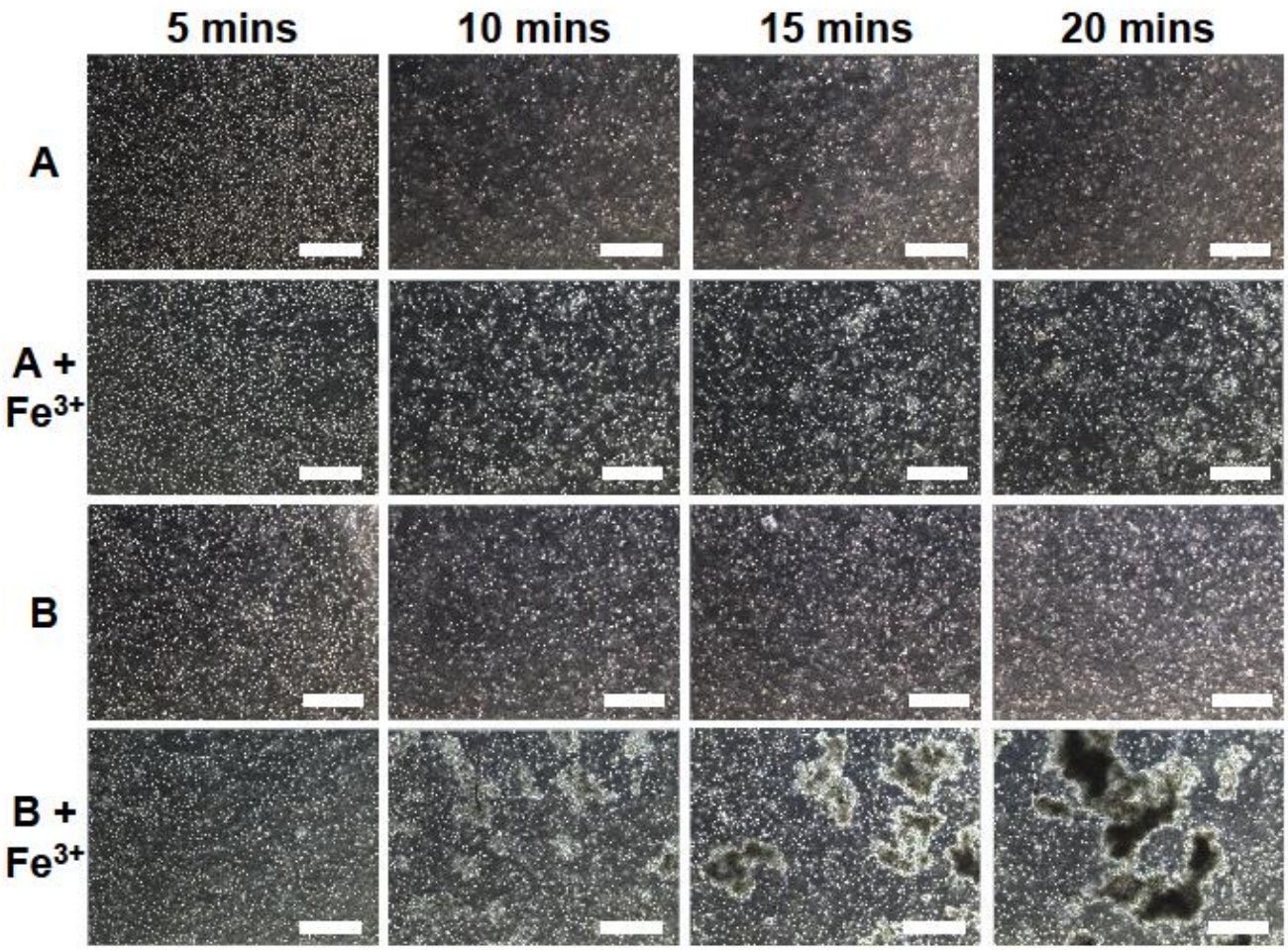


Fig. S1.1 HT29 cell aggregation experiments – supplementary images 1. Representative images of unmodified (A) and maltol-modified (B) HT29 cells in the absence and presence of 50 μM $FeCl_3$. Scale bars = 400 μm. These are additional images to those provided in **Fig. 1**, which only showed the last series of images ($B+Fe^{3+}$) from this set of experiments.

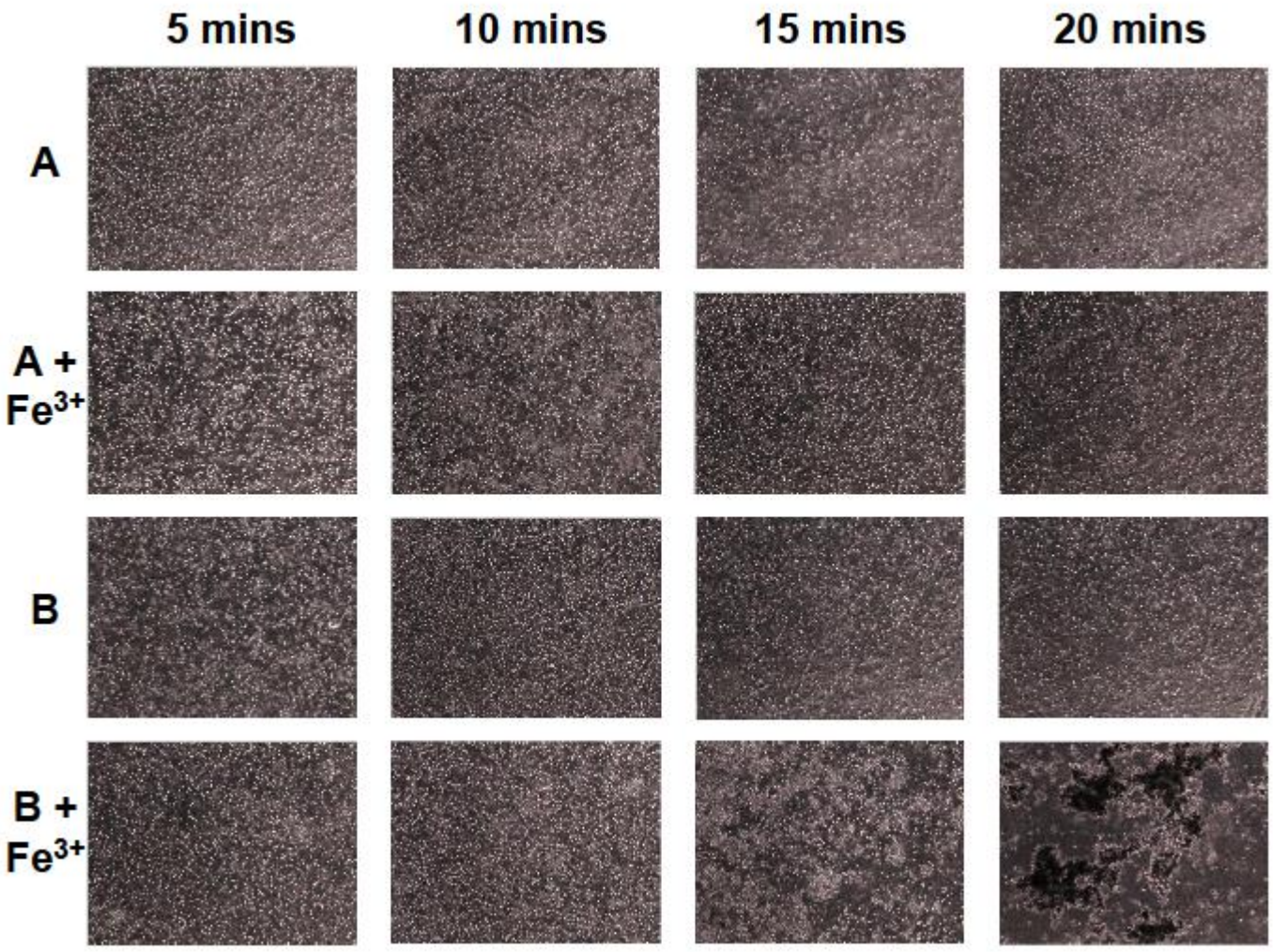


Fig. S1.2 HT29 cell aggregation experiments – supplementary images 2. Representative images of unmodified (A) and maltol-modified (B) HT29 cells in the absence and presence of 50 μM $FeCl_3$. Scale bars = 400 μm. These are additional images to those provided in **Fig. 1**.

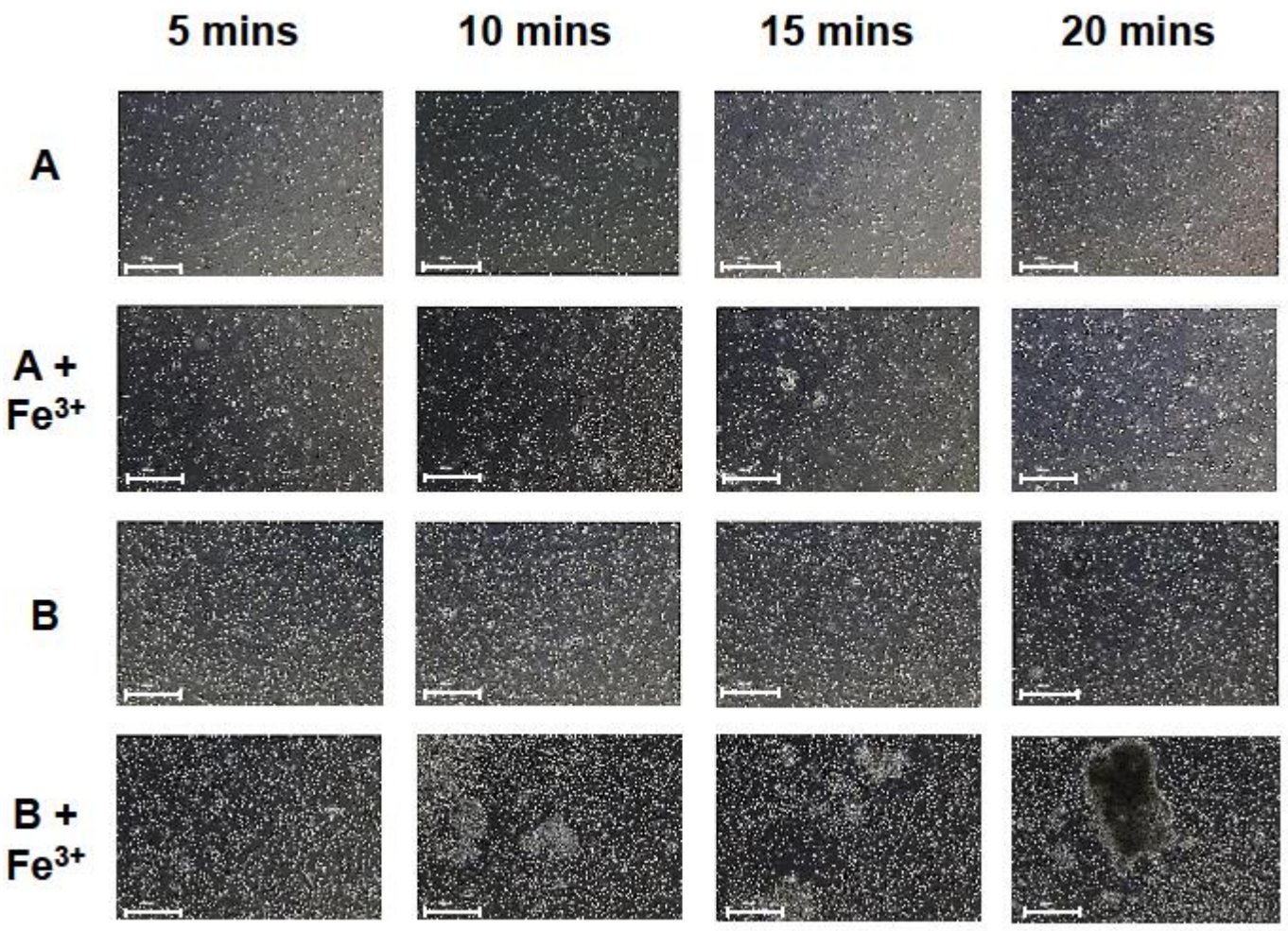


Fig. S1.3 HT29 cell aggregation experiments – supplementary images 3. Representative images of unmodified (A) and maltol-modified (B) HT29 cells in the absence and presence of 50 μM $FeCl_3$. Scale bars = 400 μm. These are additional images to those provided in **Fig. 1**.

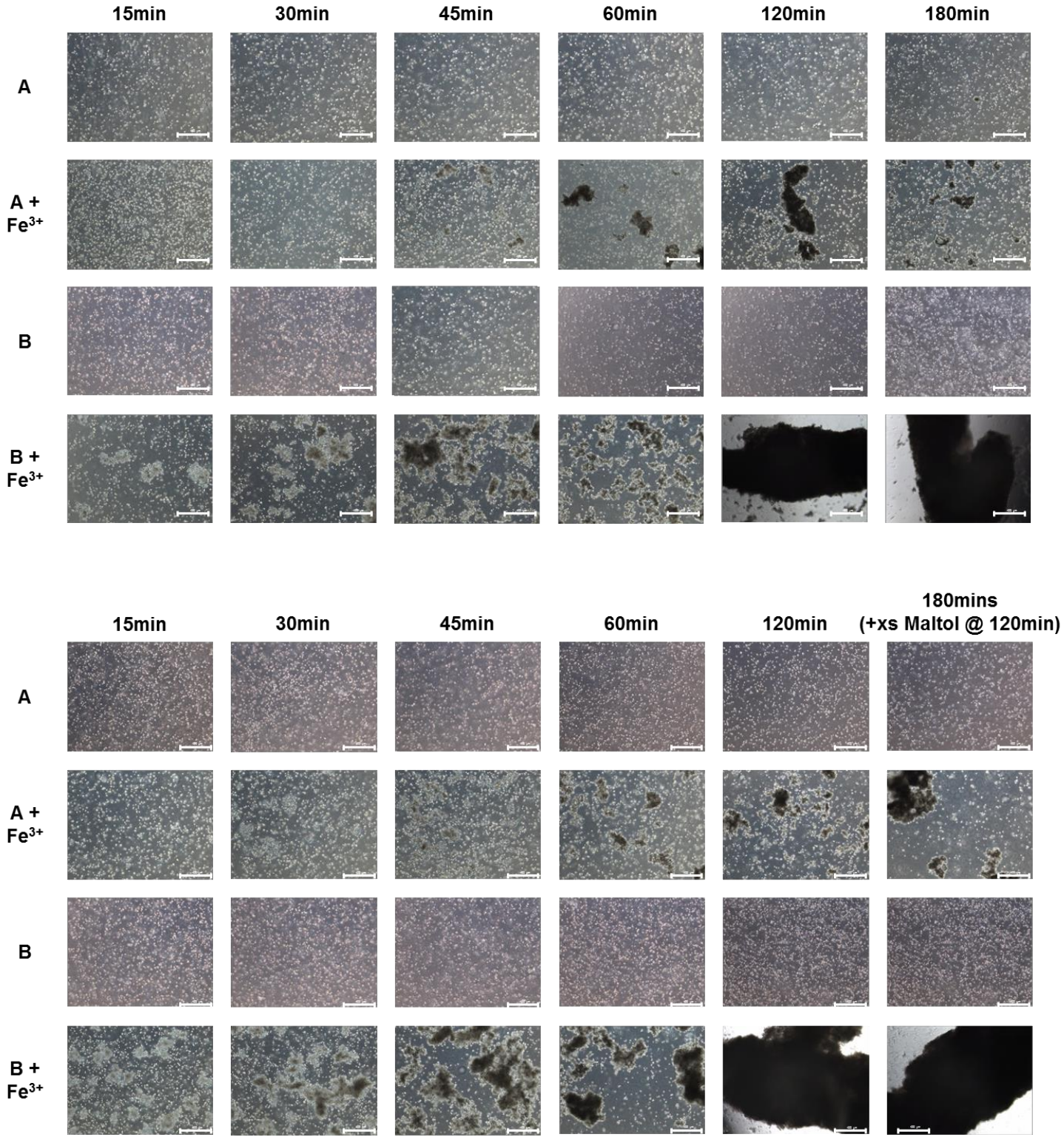


Fig. S2 Extended HT29 cell aggregation experiments. Representative images of unmodified (A) and maltol-modified (B) HT29 cells in the absence and presence of 50 μM $FeCl_3$. Cells were prepared as described above in the General Experimental, Cell Aggregation, except (point 2) the cells were incubated with a 400 μM maltol hydrazide **6** solution in PBS. +xs Maltol = a PBS solution of maltol (10-fold excess) was added at the 120min time-point and left for 60mins. The images shown are at 180mins from the start of the experiment. Scale bars = 400 μm.

Metal Screen

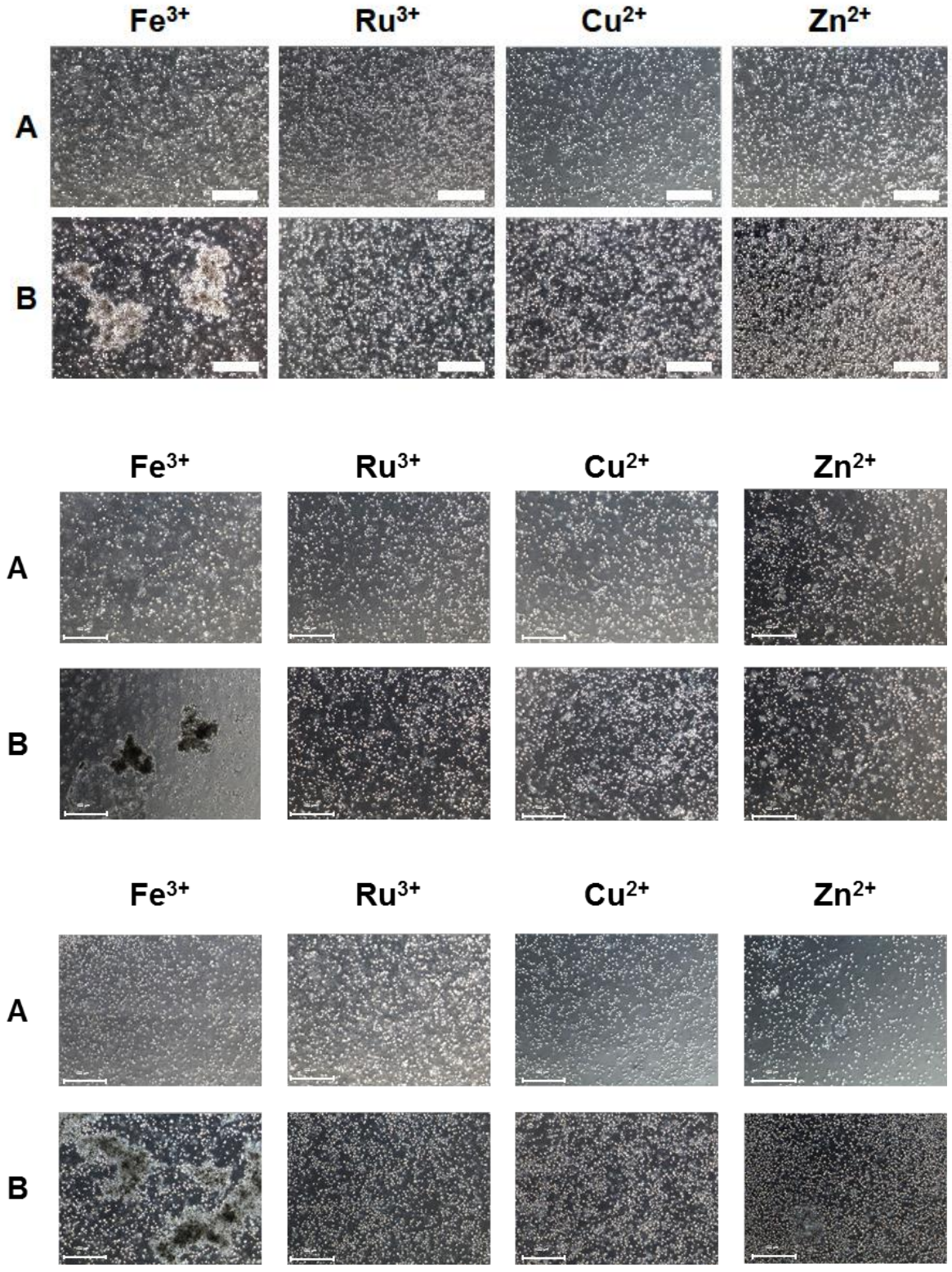


Fig. S3 Metal screen – supplementary images 1, 2 and 3. Representative images of unmodified (A) and maltol-modified (B) HT29 cells in the presence of 50 μM of MCl_x after 20 mins agitation. Scale bars = 400 μm.

HT29 and MDA-MD-231 cell Aggregation Experiments

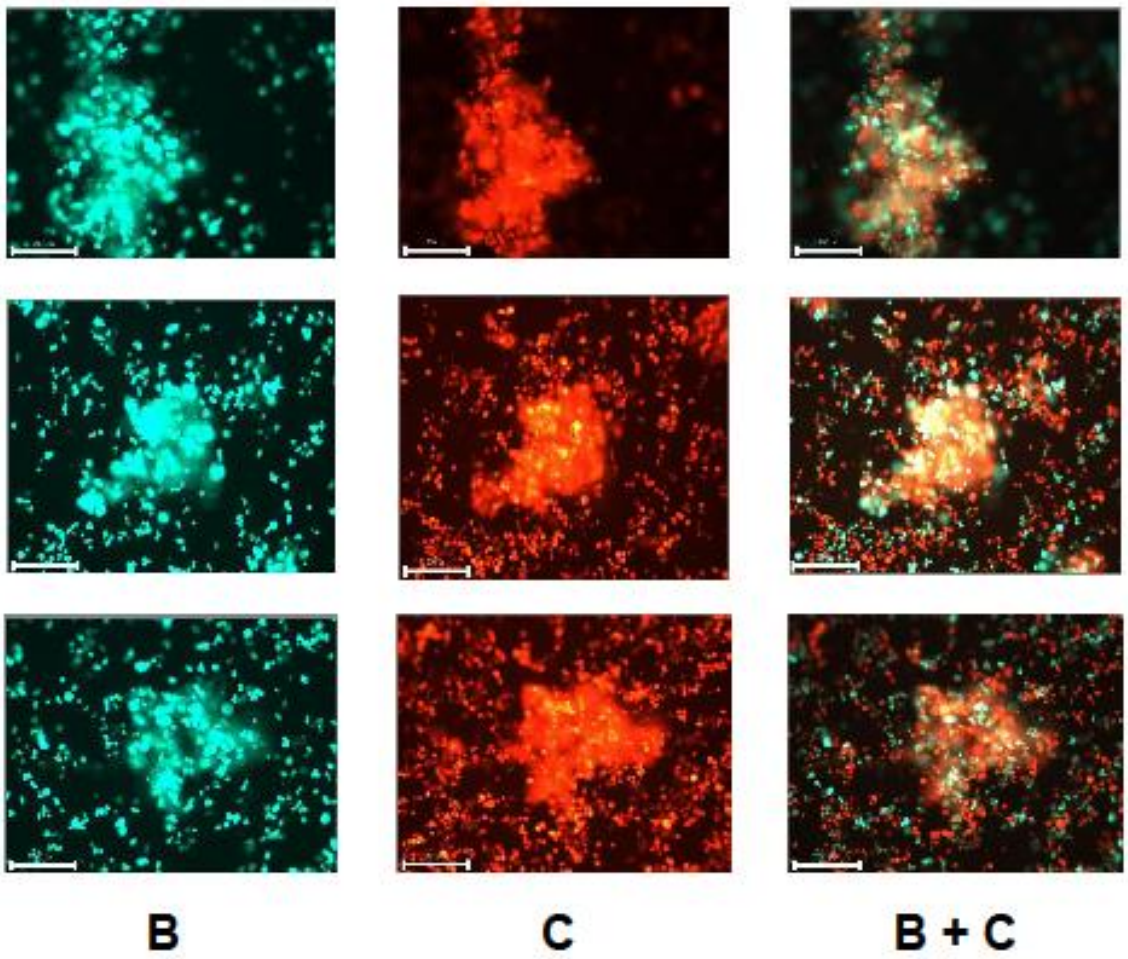


Fig. S4 HT29 and MDA-MD-231 cell aggregation experiments - supplementary images from different experiments. Representative fluorescence images of maltol-modified HT29 cells (green, B) and MDA-MB-231 cells (red, C) in the presence of 50 μM Fe^{3+} after 20 mins incubation. Scale bars = 200 μm. These are additional images to those provided in **Fig. 4**.