Supplementary Material (ESI) for Chemical Communications


# A Novel Anti-Cancer Bifunctional Platinum Drug Candidate with Dual DNA Binding and Histone Deacetylase Inhibitory Activity

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## Experimental

### Materials, methods and instrumentation

4-nitrobenzaldehyde, dimethyl malonate, piperidine, benzoic acid, malonic acid, $K_2PtCl_4$ and deuterated solvents were all purchased from Aldrich and used without further purification. Suberic anhydride[1] and iodoplatin[2] were synthesized as previously reported. IR spectra were recorded as KBr discs (4000-400 $cm^{-1}$) on a Mattson Genesis II CSI FTIR spectrometer and the spectra analysed using WinFirst software. $^1$H NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer and the spectra analysed using TopSpin 1 software. The residual undeuterated DMSO signal at 2.505 ppm or the $Me_4Si$ signal were used as internal references. Liquid chromatography-mass spectrometry experiments were performed on a Quattro Micro quadrupole electrospray mass spectrometer (Micromass, Waters Corp., USA): 10 μL of the samples were injected in 300 μL of acetonitrile:water (60:40, v/v). The mass spectrometry data were acquired both in positive and negative ion modes. Elemental analysis (C, H, N) were performed at the Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland.

### Chemical syntheses

### Synthesis of malSAHA

(**a**) 4-nitrobenzaldehyde (4.46 ml, 0.039 mol) and dimethyl malonate (4.90g, 0.032 mol) were added to piperidine (0.38 mls, 3.90 mmol) and benzoic acid (0.48g, 3.90 mmol), which were previously stirred in ethanol (30 ml) for 10 minutes. The reaction was refluxed for five hours, cooled overnight and a yellow crystalline solid (**a**) (6.80 g, 80%) collected and dried. $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 8.16 (2H, d, $^3J$ 7.0 Hz, aromatic H), 7.73 (1H, s, CH), 7.50 (2H, d, $^3J$ 7.0 Hz, aromatic H), 3.81 (3H, s, $CH_3$), 3.78 (3H, s, $CH_3$); $\delta_C$ (100 MHz; $CDCl_3$; $Me_4Si$) 166.06, 163.72, 148.50, 139.99, 139.09, 129.91, 129.23, 124.03, 53.09, 53.02.

(**b**) $NaBH_3CN$ (5.97 g, 95.0 mmol) was added to **a** (6.30 g, 23.8 mmol) in methanol (90 ml) slowly over 15 minutes. The reaction was stirred overnight at room temperature. The pH was adjusted from 10 to 2.5 using dilute HCl. Deionised water (100 ml) was added to the solution and the aqueous layer was extracted with chloroform (3 x 30 ml). The organic layer was dried with $Na_2SO_4$ and the solvent removed *in vacuo* to give a waxy yellow solid, which subsequently solidified (6.13 g, 95%). $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 8.08 (2H, d, $^3J$ 8.8 Hz, aromatic H), 7.30 (2H, d, $^3J$ 8.8 Hz, aromatic H), 3.65 (6H, s, $CH_3$), 3.61 (1H, t, $^3J$ 7.8 Hz, CH), 3.25 (2H, d, $^3J$ 7.8 Hz, $CH_2$); $\delta_C$ (100 MHz; $CDCl_3$; $Me_4Si$) 168.63, 147.03, 145.41, 129.79, 123.84, 52.86, 34.39.

**(c)** 10% Pd on activated carbon (0.63 g, wet carefully with methanol, 7 ml) was added to **b** (6.33 g, 23.7 mmol) in methanol (100 ml). The reaction was stirred under an atmosphere of hydrogen overnight at room temperature. The Pd/C was filtered and the

solvent from the filtrate was removed *in vacuo* to give **c** (4.96 g, 88%) as a brown oil. $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 6.97 (2H, d, $^3J$ 8.4 Hz, aromatic H), 6.60 (2H, d, $^3J$ 8.4 Hz, aromatic H), 3.69 (6H, s, $CH_3$), 3.59 (1H, t, $^3J$ 8.0 Hz, CH), 3.10 (2H, d, $^3J$ 8.0 Hz, $CH_2$); $\delta_C$ (100 MHz; $CDCl_3$; $Me_4Si$) 169.39, 145.05, 129.58, 127.58, 115.28, 52.51, 34.06.

(**d**) To suberic anhydride (3.16g, 20.2 mmol) dissolved in anhydrous THF (60 ml) and under an atmosphere of argon was added **c** (4.80 g, 20.2 mmol) dissolved in anhydrous THF (60 ml). The reaction was stirred overnight at room temperature. A white solid, the dianilide, was filtered and dried over $P_2O_5$. Deionised water (60 ml) was added to the filtrate, which was subsequently extracted with dichloromethane (3 x 30 ml). The organic layer was dried with $Na_2SO_4$ and the solvent removed *in vacuo* to give a waxy solid, which was recrystallised from ethyl acetate to give **d** (5.51 g, 67%). $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 7.35 (2H, d, $^3J$ 8.4 Hz, aromatic H), 7.15 (1H, s, N-H), 7.06 (2H, d, $^3J$ 8.4 Hz, aromatic H), 3.64 (6H, s, $CH_3$) 3.55 (1H, t, $^3J$ 8.0 Hz, CH), 3.10 (2H, d, $^3J$ 8.0 Hz, $CH_2$), 2.24 (4H, m, $CH_2$), 1.62 (2H, p, $^3J$ 7.2 Hz, $CH_2$), 1.55 (2H, p, $^3J$ 7.2 Hz, $CH_2$), 1.31 (4H, m, $CH_2$); $\delta_C$ (100 MHz; $CDCl_3$; $Me_4Si$) 178.02, 171.31, 171.23, 169.20, 129.37, 119.95, 60.43, 53.34, 52.62, 37.54, 34.18, 33.62, 28.72, 28.62, 25.31, 24.44, 21.08, 14.21.

**(**e**)** To d (1.93 g, 4.93 mmol) in anhydrous THF (40 ml) and under an atmosphere of argon was added ethyl chloroformate (0.63 ml, 6.55 mmol) and triethylamine (0.99 ml, 7.09 mmol). The mixture was stirred for 30 minutes, to which $NH_2OH$, generated from $NH_2OH.HCl$ (0.52 g, 8.18 mmol) and sodium methoxide (1.77 ml, 8.18 mmol) in dry methanol (10 ml), was added. The reaction was stirred for 24 hours. A white solid was filtered and discarded. The solvent from the filtrate was removed *in vacuo*. Deionised water (30 ml) was added to a waxy solid and extracted with ethyl acetate (3 x 30 ml). The organic layer was dried with $Na_2SO_4$ and the solvent removed *in vacuo* to give a yellow solid, which was recrystallised from ethyl acetate to give **e** as a white solid (1.2 g, 59%). $\delta_H$ (400 MHz; DMSO) 10.34 (1H, s, br, hydroxamic OH), 9.83 (1H, s, br, amide N-H), 8.68 (1H, s, br, hydroxamic N-H), 7.47 (2H, d, $^3J$ 8.6 Hz, aromatic H), 7.10 (2H, d, $^3J$ 8.6 Hz, aromatic H), 3.80 (1H, t, $^3J$ 7.8, CH), 3.60 (6H, s, $CH_3$), 3.01 (2H, d, $^3J$ 7.8 Hz, $CH_2$), 2.25 (2H, t, $^3J$ 7.2 Hz, $CH_2$), 1.91 (2H, t, $^3J$ 7.2 Hz, $CH_2$), 1.52 (2H, p, $^3J$ 7.2 Hz, $CH_2$), 1.44 (2H, p, $^3J$ 7.2 Hz, $CH_2$), 1.25 (4H, m, $CH_2$); $\delta_C$ (100 MHz; DMSO) 171.07, 169.03, 168.82, 137.86, 131.93, 128.87, 118.98, 52.77, 52.30, 36.29, 33.53, 32.20, 28.37, 25.00.

**malSAHA** To **e** (1.14 g, 2.79 mmol) dissolved in methanol (40 ml) was added NaOH (0.88 g, 22.32 mmol) dissolved in deionised water (10 ml). the reaction was stirred at 55 $^0$C for three hours. The pH of the solution was adjusted from 9 to 3 and the reaction mixture was concentrated *in vacuo* to give **malSAHA** (0.42 g, 40%) as a white precipitate (Found: C, 56.5; H, 6.3; N, 7.0%. $C_{18}H_{24}N_2O_7$ requires C, 56.8; H, 6.4; N, 7.05%); $\nu_{max}$(KBr)/cm$^{-1}$ 3310 br, 3231 br, 3067, 2928, 2858 s, 1712 vs, 1657 vs, 1606vs; $\delta_H$ (400 MHz; d$^6$ DMSO) 12.69 (2H, br, Carboxylic OH), 10.34 (1H, s, br, hydroxamic OH), 9.82 (1H, s, amide N-H), 8.67 (1H, s, br, hydroxamic N-H), 7.46 (2H, d, $^3J$ 8.5 Hz, aromatic H), 7.11 (2H, d, $^3J$ 8.5 Hz, aromatic H), 3.50 (1H, t, $^3J$ 8.0 Hz, CH), 2.95 (2H, d, $^3J$ 7.8 Hz, $CH_2$), 2.24 (2H, t, $^3J$ 7.25 Hz, $CH_2$), 1.91 (2H, t, $^3J$ 7.25 Hz, $CH_2$), 1.52 (2H, p, $^3J$ 7.0 Hz, $CH_2$), 1.46 (2H, p, $^3J$ 7.0 Hz, $CH_2$), 1.25 (4H,

m, $CH_2$); $\delta_C$ (100 MHz; DMSO) 171.02, 170.48, 169.05, 137.61, 132.99, 128.89, 118.90, 53.20, 36.28, 33.64, 32.19, 28.37, 25.00. ESI-MS *m/z*: 381.2 ($[M+H]^+$).

**Synthesis of platinum complexes**

***cis*-[Pt(NH$_3$)$_2$(malSAHAH$_{-2}$)].3H$_2$O**

Iodoplatin (0.3 g, 0.62 mmol) and $AgNO_3$ (0.21 g, 1.21 mmol) in deionised water (15 ml) were stirred overnight in the dark. The insoluble AgI was filtered off and malSAHA (0.26 g, 0.68 mmol) dissolved in an NaOH solution (0.055g, 1.36 mmol NaOH in 5 ml $H_2O$) was added to the filtrate. The reaction was stirred at room temperature for 3 days. A buff coloured solid (0.24 g, 66%) was filtered off and dried over $P_2O_5$ (Found: C, 32.83; H, 4.79; N, 8.89%. $C_{18}H_{34}N_4O_{10}Pt$ requires C, 32.68; H, 5.18; N, 8.47%). $\nu_{max}$(KBr)/cm$^{-1}$ 3280 br, 3117 s, 1666 vs, 1654 vs, 1636 vs, 1614, 1530 vs; $\delta_H$ (400 MHz, d$^6$ DMSO) 10.33 (1H, s, br, hydroxamic OH), 9.75 (1H, s, amide N-H), 8.70 (1H, s, br, hydroxamic N-H), 7.39 (2H, d, $^3J$ 8.5 Hz, aromatic H), 7.12 (2H, d, $^3J$ 8.5 Hz, aromatic H), 4.18 (6H, s, br, $NH_3$) 3.95 (1H, t, $^3J$ 6.5 Hz, CH), 2.95 (2H, d, $^3J$ 6.5 Hz, $CH_2$), 2.24 (2H, t, $^3J$ 7.3 Hz, $CH_2$), 1.91 (2H, t, $^3J$ 7.5 Hz, $CH_2$), 1.52 (2H, p, $^3J$ 7.0 Hz, $CH_2$), 1.46 (2H, p, $^3J$ 6.75 Hz, $CH_2$), 1.26 (4H, m, $CH_2$). ESI-MS *m/z*: 606.2 ($[M+H]^-$).

***cis*-[Pt(NH$_3$)$_2$(malH$_{-2}$)]**

Iodoplatin (0.20 g, 0.41 mmol) and $AgNO_3$ (0.14 g, 0.81 mmol) in deionised water (10 ml) were stirred overnight in the dark. The insoluble AgI was filtered off and malonic acid (0.05 g, 0.46 mmol) was added to the filtrate. The pH of the solution was adjusted from 3.5 to 6.3 using dil. NaOH. The reaction was stirred at room temperature for 24 hours. A grey/white coloured solid (0.11 g, 58%) was filtered, washed with cold $H_2O$ and dried over $P_2O_5$ (Found: C, 10.78; H, 2.49; N, 8.13%. $C_3H_8N_2O_4Pt$ requires C, 10.88; H, 2.43; N, 8.46%); $\nu_{max}$(KBr)/cm$^{-1}$ 3282, 3266, 3126 s, 1655 vs, 1625 vs, 1582 vs; $\delta_H$ (400 MHz, d$^6$ DMSO): 4.17 (6H, s, br, $NH_3$), 3.20 (2H, s, $CH_2$). ESI-MS *m/z*: 330.0 ($[M+H]^-$).

**Unwinding of Negatively Supercoiled DNA**

DNA unwinding was examined by electrophoretic mobility shift assays. Samples of pUC19 plasmid DNA (30μM), purchased form Fermentas, were incubated with increasing concentrations of *cis*-[Pt(NH$_3$)$_2$(malSAHAH$_{-2}$)] (0-100μM range) or cisplatin at 37 $^o$C in the dark for 72 h. All samples were subjected to electrophoresis on 1% (w/v) agarose gel run at 100 V in TAE buffer at 25 $^o$C. The gels were then stained with 0.5μg/ml EtBr, visualised using a UVP transilluminator and photographed. Initial experiments were conducted over a broad range of drug to nucleotide ratios and subsequent experiments were performed using a narrower concentration range. The experiments described were undertaken three times to ensure reproducibility.

**Histone Deacetylase Inhibitory Activity**

The ability of the test compounds to inhibit HDAC1 was investigated in triplicate using Cayman's HDAC1 Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, USA) according to the manufacturer's instructions. Trichostatin A was used as a positive control. The assay provides a fast two-step fluorescence-based method for measuring HDAC activity. Briefly, in the first step an acetylated lysine residue was incubated with HDAC1 and potential inhibitors. Deacetylation sensitises the substrate

so that treatment with the HDAC developer in the second step releases a fluorescent product. The fluorescent reaction product was analysed using a plate reader with an excitation wavelengths of 350 nm and emission wavelengths of 460 nm. Stock solutions of investigated $Pt^{II}$ complexes and Trichostatin A, SAHA and malSAHA were prepared freshly in buffer and DMSO respectively and diluted to various working concentrations. The $IC_{50}$ values defined as the drug concentration which inhibits HDAC1 activity by 50%, were determined by graphing percentage of initial activity (control, no inhibitor) as a function of inhibitor concentration.

## Cell Lines and Culture Conditions

A2780P and A2780cisR human ovarian carcinoma cells (obtained from the European Collection of Cell Cultures) were cultured in RPMI 1640 supplemented with 10% Foetal Bovine Serum (Biosera, East Sussex, UK), 2 mM Glutamine (EuroClone, Wheterby, UK) and 100 U/mL penicillin and 100 μg/mL streptomycin (EuroClone, Wheterby, UK). In order to retain resistance 1 μM cisplatin was added to the media of the A2780 cisR cells every third passage.

Normal Neonatal Human Dermal Fibroblast**,** NHDF, cells were cultured in Fibroblast Basal Medium (FBM®, Clonetics, Walkersville, USA) supplemented with hFGF-B, insulin, 5% FBS and gentamicin/amphotericin-B (FGM®-2 SingleQuots, Clonetics, Walkersville, USA) and used at low passage numbers.

All cells were kept in a humidified atmosphere with 5% $CO_2$ at 37°C; cells from confluent monolayers were removed from flasks by trypsin/EDTA solution and their viability determined by the trypan blue exclusion test.

## *In vitro* Cytotoxicity Assay

Stock solutions of $Pt^{II}$ complexes and ligands were freshly prepared in the appropriate culture medium and diluted with culture medium to various working concentrations.
Cell growth was determined by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, (MTS assay, CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Southampton, UK), a colorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt to blue formazan. 10,000 ovarian cancer cells or 3,000 fibroblast cells were seeded per well onto 96-well plates in 100 μl of the appropriate culture medium. 24 hr after seeding the cells, the media was removed and the cells were treated with 100 μl of media containing the test compound. A range of concentrations between 1 μM and 100 μM was used. After 72 hr of treatment, 20 μl of the MTS reagent was added to each well and the plates incubated for 2 hr at 37°C. The absorbance was measured at 490 nm using a Wallac 1420 Victor 3V plate reader (Perkin-Elmer Life Sciences, Boston, USA). The percentages of surviving cells relative to untreated controls were determined. The $IC_{50}$ values defined as the drug concentration which inhibits cell growth by 50% were estimated graphically from dose-response plots.

1. A. Mai, M. Esposito, G. Sbardella and S. Massa, *Org. Prep. Proced. Int.*, 2001, **33**, 391-394.
2. S. C. Dhara, 1970, **8**, 193-194.