A novel platinum complex of the histone deacetylase inhibitor belinostat; rational design, development and *in vitro* cytotoxicity

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Abstract

The successful design and synthesis of a novel Pt complex of the histone deacetylase inhibitor belinostat is reported. Molecular modelling assisted in the identification of a suitable malonate derivative of belinostat (mal-p-Bel) for complexation to platinum. Reaction of [Pt(NH$_3$)$_2$(H$_2$O)$_2$](NO$_3$)$_2$ with the disodium salt of mal-p-Bel gave cis-[Pt(NH$_3$)$_2$(mal-p-Bel)$_2$H] in excellent yield. An *in vitro* cytotoxicity study revealed that cis-[Pt(NH$_3$)$_2$(mal-p-Bel)$_2$H] possesses (i) considerable cytotoxicity against the reported ovarian cancer cell lines, (ii) enhanced cytotoxicity as compared to the previously reported Pt histone deacetylase inhibitor conjugate, cis-[Pt$_{III}$N(H$_3$)$_2$(malSAHA)$_2$H] and (iii) favourable cyto-selective properties as compared to cisplatin and belinostat.
1. Introduction

Over the past 30 years platinum (Pt) compounds have played a very important and well documented role in treating cancer.[1, 2] Currently nearly 50% of all anti-cancer therapies are Pt-based[3] and three Pt compounds are in worldwide clinical use, namely cisplatin, carboplatin and oxaliplatin (Figure 1).[1, 2] The cytotoxicity of Pt drugs is attributed to multiple mechanisms[4] but primarily their ability to enter cells, hydrolyse and covalently bind DNA, forming Pt-DNA adducts. These events can lead to DNA damage responses and ultimately programmed cell death, apoptosis.[1, 2, 4] The clinical efficacy of Pt drugs is limited by drawbacks though, such as toxicity, but primarily by the high incidence of chemoresistance (intrinsic or acquired).[1, 2, 4] While metal complexes continue to play a crucial role in anti-cancer treatment, strategies to circumvent problems associated with resistance involve the use of combination therapies or the development of novel drug candidates that target at least two distinct mechanisms.[1, 4]

![Figure 1 Structures of cisplatin, carboplatin and oxaliplatin.](image)

We are interested in combining platinum compounds with histone deacetylase (HDAC) inhibitors (HDACIs) so as to target a broader spectrum of human cancers than clinically used Pt drugs.[5-7]

Histones are the main protein component of chromatin, around which DNA coils. HDACs are a class of zinc metalloenzymes that deacetylate core histone lysine residues, which results in a condensed chromatin structure and ultimately transcriptional repression.[8] Inhibition of HDAC function therefore dramatically affects chromatin structure and thus function. More recently HDACIs have been shown to target non-histone proteins involved in cellular proliferation, migration, death, DNA repair, angiogenesis, inflammation and the immune response.[9, 10] A range of structurally diverse HDACIs have been shown to be effective anti-cancer (cytostatic and cytotoxic) agents via multiple mechanisms, including inducing cell-cycle arrest, intrinsic and extrinsic apoptotic mechanisms, mitotic cell death, autophagic cell death, generation of reactive oxygen species, inhibiting angiogenesis, and improving
NK cell–mediated tumour immunity.[8-10] In contrast HDACIs have little effect on normal cells and therefore many of these inhibitors are now undergoing clinical trials.[10]

Suberoylanilide hydroxamic acid (SAHA, vorinostat), (Figure 3) was the first FDA-approved pan-HDACI to enter the clinic as a treatment for cutaneous T-cell lymphoma[11]. It is well tolerated in patients, particularly at doses which exhibit a potent anti-cancer effect.[12, 13] We previously developed a novel anti-cancer bifunctional Pt drug candidate, cis-[Pt(NH$_3$)$_2$(malSAHA$_{2H}$)] (Figure 2), with dual DNA binding and HDAC inhibitory activity and which exhibited selectivity for cancer cells over normal cells.[5, 7] Furthermore, we developed novel trans-platinum complexes of the HDACI, valproic acid.[14]

![Figure 2 Structures of HDACI's, SAHA, belinostat and p-Bel and Pt-HDACI conjugates, cis-[Pt(NH$_3$)$_2$(malSAHA$_{2H}$)] and trans-[Pt(VPA$_{1H}$)$_2$(NH$_3$)(py)].](image)

With a view to developing a Pt-HDACI conjugate with enhanced cytotoxicity as compared to cis-[Pt(NH$_3$)$_2$(malSAHA$_{2H}$)] for example, we recognized the potential of belinostat (Figure 2), an HDACI and an experimental drug candidate currently in clinical trials for the treatment of hematological malignancies and solid tumors.[15] Belinostat was chosen for a comprehensive study due to its extensively documented lower cell cytotoxic IC$_{50}$ values when compared to that of SAHA and the
likelihood that it will be the third HDACI to be approved for clinical use.[15] Though the arylsulfonamide group in belinostat is meta substituted we selected the para analogue (p-Bel, Figure 2) for our study on the basis that it has comparable HDAC inhibitory and anti-proliferative activity as compared to belinostat but its synthesis is more facile.[16]

We sought to derivatise p-Bel with a dicarboxylate linker so as to facilitate its binding to and hydrolysis from a cis-diammine Pt(II) moiety. We utilised molecular modelling to identify which p-Bel to develop with a view to facilitating complexation to Pt while minimising any negative impact on HDAC inhibitory activity. We anticipated that the dual functioning Pt-p-Bel conjugate would hydrolyse in much the same way as carboplatin in cells[17, 18] by releasing the p-Bel derivative to inhibit HDACs and the cytotoxic cis-diammine Pt moiety to bind DNA nucleobases.

Herein we describe the rational design, development, characterisation and in vitro cytotoxic activity of a novel Pt-p-Bel conjugate, cis-[Pt(NH$_3$)$_2$(mal-p-Bel$_{2H}$)], against cisplatin sensitive and resistant ovarian cancer cell lines (A2780 and A2780 cisR) and normal human dermal fibroblasts (NHDFs).

2. Experimental

2.1 Molecular modelling

The X-ray crystal structure of SAHA bound to HDAC (PDB:1T69) was used to dock belinostat, p-Bel and dicarboxylate derivatives of p-Bel. The protein structure was prepared for docking using the MOE suite of programme (Chemical Computing Group, Montreal). Correct protonation of the active site was achieved using the protonate3D function in MOE. AutoDockTools (Scripps) was used to prepare the protein and ligand structures in PDBQT format. Ligands were docked to the HDAC target using Vina with default settings, exhaustiveness = 8 and a 22,437 Å$^3$ binding box.[19] Analysis of the docked poses was achieved using MOE.

2.2 Materials and instrumentation
Cinnamic acid, chlorosulphonic acid, oxalyl chloride, hydroxylamine hydrochloride and deuterated solvents were all purchased from Sigma Aldrich and used without further purification. 2-(4-Amino-benzyl)-malonic acid dimethyl ester[5] and iodoplatin[20] were synthesized as previously reported.

$^1$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 DMF at 8.03 ppm 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 was acquired both in positive and negative ion modes. Elemental analysis (C, H, N) was performed at the Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland.

2.3 Cell lines and cell culture

Two human-derived malignant ovarian cell lines (A2780 and A2780 cisR) were used to determine the in vitro anti-cancer chemotherapeutic potential of test agents. A2780 cisR cells are a cisplatin-resistant human ovarian cell line which were developed by chronic exposure of the parent cisplatin-sensitive A2780 cell line to increasing concentrations of cisplatin.[21] Additionally, these cells are cross-resistant to melphalan, adriamycin and irradiation. In order to maintain their resistant phenotype, cells were exposed to cisplatin (1 µM) every third passage. Both cell lines were maintained in RPMI-1640 media with Earle’s balanced salt solution (EBSS), containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % (v/v) foetal bovine serum (FBS).

Normal human dermal fibroblast (NHDF) primary cells were used to determine the in vitro cytoselective nature of test agents, compared to the malignant ovarian cell lines mentioned above. These 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 cultured at 37 °C in a humidified atmosphere, containing 5 % (v/v) CO$_2$ and were included in experiments while in the mid-log phase of growth.
2.4 Syntheses

2.4.1 Synthese of mal-p-Bel

2.4.1.1 Synthesis of precursor 1

Cinnamic acid (16.00 g, 0.107 mol) was added slowly to neat chlorosulphonic acid (60 mL) at 0 ºC. The mixture was stirred at 0 ºC for 3 hours and then at room temperature for three days. The dark viscous syrup was slowly poured onto ice affording a white precipitate which was filtered, washed with water and recrystallized in small portions from dioxane to afford 1 as white crystals. Yield: 2.73 g, 34 %. Found C, 44.10; H, 3.01%. C₉H₇ClO₄S requires C, 43.82; H, 2.86%. δ_H (400 MHz, d⁶ DMSO): 13.80 (1H s), 7.62 (2H, d, J 8.5 Hz), 7.60 (2H, d, J 8.5 Hz), 7.54 (1H, d, J 16.0 Hz), 6.53 (1H, d, J 16.0 Hz). δ_C (100 MHz, d⁶ DMSO): 167.43, 149.68, 143.28, 134.27, 127.97, 125.62, 119.57. ESI-MS m/z: 245 (calculated 245) ([M-H]⁺).

2.4.1.2 Synthesis of the precursor 2

1 (2.25g, 9.15 mmol) was added to a solution of 2-(4-amino-benzyl)-malonic acid dimethyl ester, (5.0g, 18.30 mmol) and pyridine (3 ml) in DCM (30 ml). The resultant solution was stirred at 40 ºC for 1 hr. The solution was cooled to room temperature after which 6M HCl was slowly added resulting in the formation of a pink emulsion. The liquid was decanted off and the solid dissolved in EtOAc which was then washed with 6M HCl (x2), water and brine before being dried over Na₂SO₄. The solvent was evaporated off in vacuo to afford pale pink powder. Yield: 3.18 g, 35%. Found C, 56.34; H, 4.80; N, 3.08%. C₂¹H₂¹NO₈S requires C, 56.37; H, 4.73; N, 3.13%. δ_H (400 MHz, d⁶ DMSO): 12.70 (1H, s), 10.36 (1H, s), 7.90 (2H, d, J 8.5 Hz), 7.75 (2H, d, J 8.5 Hz), 7.65 (1H, d, J 16.0 Hz), 7.10 (2H, d, J 8.5 Hz), 7.00 (2H, d, J 8.5 Hz), 6.65 (1H, d, J 16.0 Hz), 3.85 (1H, t, J 8.50 Hz), 3.61 (6H, s), 3.02 (2H, d, J 16.0 Hz). δ_C (100 MHz, d⁶ DMSO): 168.73, 167.10, 141.81, 140.22, 138.35, 133.51, 129.51, 128.74, 122.36, 120.67, 52.51, 52.23, 33.36. ESI-MS m/z: 447.1 (calculated 447.1) ([M-H]⁺).
2.4.1.3 Synthesis of the precursor 3

To a suspension of 2 (0.487, 1.12 mmol) in 10 ml dry DCM, was added a 2M solution of oxalyl chloride in DCM (0.674 ml, 1.34 mmol) and two to three drops of DMF. The mixture quickly formed an off green solution which was refluxed for 1 h. The solvent was evaporated and the residue was dried in vacuo and redissolved in 10 ml dry THF, to which NH\textsubscript{2}OH, generated from reaction of hydroxylamine hydrochloride (0.31g, 4.49 mmol) in dry MeOH (4 ml) and a 4.63 M solution of NaOMe in MeOH (0.970 ml, 4.49 mmol) was added. The resultant solution was stirred at room temperature for 1 hour. The mixture was partitioned between ethyl acetate and 2M HCl. The organic layer was extracted, washed with water, then brine and further dried over Na\textsubscript{2}SO\textsubscript{4}. The solvent was evaporated off in vacuo to afford light off-white/yellow solid. Yield: 1.47 g, 62%. Found C, 52.54; H, 4.89; N, 5.88%. C\textsubscript{21}H\textsubscript{22}N\textsubscript{2}O\textsubscript{8}S.H\textsubscript{2}O requires C, 52.49; H, 5.03; N, 5.83%. δ\textsubscript{H} (400 MHz, d\textsuperscript{6} DMSO): 10.88 (1H, s), 10.27 (1H, s), 9.15 (1H, s), 7.70 (4H, q, J 8.5 Hz), 7.50 (1H, d, J 16.0 Hz), 7.01 (2H, d, J 8.5 Hz), 6.98 (2H, d, J 8.5 Hz), 6.53 (1H, d, J 16.0 Hz), 3.78 (1H, t, J 8.5 Hz). δ\textsubscript{C} (100 MHz, d\textsuperscript{6} DMSO): 168.73, 162.00, 139.58, 138.35, 136.43, 135.94, 133.48, 129.51, 128.02, 127.09, 122.22, 120.67, 52.51, 52.23, 33.36. (ESI-MS m/z: 462.1 (calculated 462.1) ([M-H]\textsuperscript{+}).

2.4.1.4 Synthesis of mal-p-Bel (4)

To a solution of 3 (1.44 g, 3.00 mmol) in 48 mL of MeOH, was added a 1 M solution of NaOH (16 mL, 16 mmol) and the solution was stirred at 75 °C for 1 hr. The solution was partitioned between ethyl acetate and 2M HCl. The organic layer was extracted and washed successively with water and then brine and subsequently dried over Na\textsubscript{2}SO\textsubscript{4}. The solvent was evaporated off in vacuo to afford mal-p-Bel (4) as a light off white/yellow solid. Yield: 1.00 g, 74%. Found C, 51.11; H, 4.52; N, 5.51%. C\textsubscript{19}H\textsubscript{22}N\textsubscript{2}O\textsubscript{9.5}S requires C, 51.35; H, 4.80; N, 5.84%. IR ν\textsubscript{max} (cm\textsuperscript{-1}, br = broad, s = strong, vs = very strong) 3491br, 3243br, 2924br, 1703s (carboxylate C=O), 1656vs and 1612vs (hydroxamate C=O). δ\textsubscript{H} (400 MHz, d\textsuperscript{6} DMSO): 12.72 (2H, s), 10.88 (1H, s), 10.26 (1H, s), 9.15 (1H, s), 7.69 (4H, 2 x d, J 8.5 Hz), 7.45 (1H, d, J 16.0 Hz), 7.08 (2H, d, J
8.5 Hz), 6.97 (2H, d, J 8.5 Hz), 6.51 (1H, d, J 16.0 Hz), 3.47 (1H, t, J 8.0), 2.90 (2H d, J 8.0 Hz). δ (100 MHz, d<sup>6</sup> DMSO): 170.13 (2 x C), 162.01, 139.70, 138.95, 136.49, 135.69, 134.47, 129.48 (2 x C), 128.07 (4 x C), 122.21, 120.32 (2 x C), 53.23, 33.44. ESI-MS m/z: 433.1 (calculated 433.1) ([M-H]−).

2.2.5 Synthesis of cis-[Pt(NH<sub>3</sub>)<sub>2</sub>(mal-p-Bel-2H<sub>2</sub>)]·1.5H<sub>2</sub>O

Iodoplatin (0.30g, 0.62 mmol) and AgNO<sub>3</sub> (0.21g, 1.21 mmol) in deionised water (15 ml) were stirred for three hours in the dark at 55 °C. The insoluble AgI was filtered off. To the filtrate, mal-p-Bel (0.307 g, 0.68 mmol) dissolved in NaOH (0.055g, 1.36 mmol) in 5 ml water was added and stirred at room temperature for 3 days. The light yellow precipitate was filtered, washed with ethanol and diethylether and dried over P<sub>2</sub>O<sub>5</sub>, to afford cis-[Pt(NH<sub>3</sub>)<sub>2</sub>(mal-p-Bel-2H<sub>2</sub>)] as a yellow solid. Yield: 82%. Found C, 31.83; H, 3.52; N, 7.88%. C<sub>19</sub>H<sub>22</sub>N<sub>4</sub>O<sub>8</sub>PtS·1.5H<sub>2</sub>O requires C, 32.07; H, 3.54; N, 7.87%. IR ν<sub>max</sub> (cm<sup>−1</sup>) 3232s (hydroxamate NH), 2928s (hydroxamate OH), 1652 (carboxylate and hydroxamate C=O), 1620br (hydroxamate C=O). δ<sub>H</sub> (400 MHz, d<sup>7</sup> DMF): 11.00 (1H, s), 10.11 (1H, s), 9.45 (1H, s), 7.84 (2H, d, J 8.5 Hz), 7.76 (2H, d, 8.5 Hz), 7.51 (1H, d, J 16.0 Hz), 7.19 (2H, d, J 8.5 Hz), 7.08 (2H, d, J 8.5 Hz), 6.69 (1H, d, J 16.0 Hz), 4.44 (6H, s, br), 3.90 (1H, t, J 6.7 Hz), 3.11 (2H d, J 6.70 Hz). δ<sub>C</sub> (100 MHz, d<sup>7</sup> DMF): 175.65 (2 x C), 162.01, 140.73, 139.66, 138.68, 135.76, 130.17 (2 x C), 128.33 (2 x C), 127.80 (2 x C), 122.66, 120.68 (2 x C), 59.94, 34.16. δ<sub>Pt</sub> (64 MHz, d<sup>7</sup> DMF): -1921.31. ESI-MS m/z: 660.1 ([M+H]<sup>+</sup>) (calculated 660.1)

2.3 In vitro cytotoxicity

Ovarian cancer cells (A2780 and A2780 cisR) and normal (NHDF) cells were seeded at a density of 2.4 x 10<sup>4</sup> and 3 x 10<sup>4</sup> cells/ml respectively into sterile 96 well flat-bottomed plates (Sarstedt) and allowed to adhere overnight in 5% (v/v) CO<sub>2</sub> at 37 °C. Test agents were dissolved in dimethyl formamide (DMF) and diluted with culture media. The maximum percentage of DMF present in all wells was 0.5% (v/v). Solutions of each test agent (100 µl) were added to replicate wells in the concentration range 0-100 µM and incubated
for 72 hr. A miniaturised viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was carried out according to the method described by Mosmann (1983). The IC\textsubscript{50} value, defined as the test agent concentration causing a 50\% reduction in cellular viability, was calculated for each test compound. Each assay was carried out using five replicates per test concentration and repeated on three separate occasions. Viability was calculated as a percentage of solvent-treated (0.5 \% v/v) control cells, and expressed as a percentage of control. The statistical significance of any reduction in cellular viability was determined using one-way ANOVA (analysis of variance). A probability of 0.05 or less was deemed statistically significant.

3. Results and discussion

3.1 Molecular Modelling

We sought to derivatise the terminal benzene ring of \textit{p}-Bel with a malonate group \textit{via} a methylene group or methylene groups so as to facilitate its binding to and hydrolysis from a \textit{cis}-diammine Pt(II) moiety (Figure 3). Molecular modelling was used to identify (i) where best to derivatise the terminal benzene ring (\textit{ortho, meta or para}) of \textit{p}-Bel and (ii) whether to use one, two or three methylene spacer groups to link the malonate to the terminal benzene ring.

The X-ray crystal structure of SAHA bound to HDAC (PDB:1T69) was used to dock belinostat, \textit{p}-Bel and 9 potential dicarboxylate derivatives of \textit{p}-Bel (Figure 3). A key feature of the SAHA-HDAC crystal structure is the active site channel with the catalytic zinc ion bound by Asp267, Asp178, His180 and the hydroxamate oxygens of SAHA (Figure 4 a). The SAHA hydroxamate is further stabilised in the active site by Tyr306 and His142. The benzene rings of Phe152 and Phe208, as well as the methyl terminus of Met274, create a hydrophobic collar at the mouth of the channel that binds the aliphatic chain of SAHA. Docking of belinostat to PDB:1T69 shows an almost identical set of interactions, the key features of which are the salt bridge between the hydroxamate and catalytic site zinc atom and the hydrophobic channel (Figure 4 b).

Of the 9 potential derivatives of \textit{p}-Bel investigated, Autodock Vina docking predicts that the derivative where the malonate group is linked to the terminal benzene ring of \textit{p}-Bel \textit{via} one methylene group
at the *para* position (mal-\(p\)-Bel, Figure 3) should bind HDAC (PDB:1T69) most effectively. In turn mal-\(p\)-Bel should also bind slightly more effectively than belinostat or SAHA, with binding energies of -8.4 kcal/mol, -7.5 kcal/mol and -6.7 kcal/mol, respectively (Figure 4).

**Figure 3** General structure for investigated dicarboxylate derivatives of \(p\)-Bel and structure of mal-\(p\)-Bel.

(a)  
(b)  
(c)  

**Figure 4** Ligand interactions between (a) SAHA, (b) belinostat and (c) mal-\(p\)-Bel and HDAC.

The interactions between the co-crystalised pose of SAHA in 1T69 (a) is compared to the docked poses of belinostat (b), and mal-\(p\)-Bel (d). Arrows indicate directionality of hydrogen bonds, while ionic interactions are indicated with purple dotted lines. Solvent accessibility is shown with blue spheres, while the binding site proximity contour is indicated with a grey dotted curve. Note the buried hydrophobic channel ending in
salt bridges between the hydroxamate and the catalytic zinc cation, and the new interaction between mal-\(p\)-Bel and Lys202 at the mouth of the HDAC channel.

Many of the active site interactions for mal-\(p\)-Bel are the same as that found with both SAHA and belinostat, such as the hydroxamate oxygens bound to the catalytic zinc ion, and hydrophobic contacts with the channel residues Phe152, Phe208 and Met274 (Figure 4). However, important additional salt bridge and hydrogen-bonding networks can form between Lys202 outside the active site channel and one of the carboxyl groups in mal-\(p\)-Bel (Figure 5), likely contributing to the improved binding energy noticed in the docking studies.

![Figure 5 Docking of mal-\(p\)-Bel into HDAC](image)
The docked position of belinostat is shown with green carbon atoms, while that of mal-\(p\)-Bel is shown with cyan carbon atoms. HDAC carbon atoms are coloured orange. The transparent electrostatic surface is coloured red for negative charge, blue for positive charge and white for neutral. Non-polar hydrogen atoms (white) were omitted for clarity. Note the hydroxamate chelation to the catalytic zinc atom (brown), the pi-pi stacking between Phe208 and Phe152 with benzene in mal-\(p\)-Bel, and the salt bridge between the malonate and Lys202. Oxygen atoms are red, nitrogen blue and sulphur yellow.

3.1 Synthesis of mal-\(p\)-Bel

Mal-\(p\)-Bel was synthesised in good yield and purity according to Scheme 1. This reported synthesis was inspired by a previously published synthesis of belinostat[16] where 2-(4-amino-benzyl)-malonic acid dimethyl ester was reacted with 1 instead of aniline. The sulfonyl chloride compound, 1, was reacted with 2-(4-amino-benzyl)-malonic acid dimethyl ester to afford the arylsulphonamide compound, 2. The adduct 2 was converted to its corresponding hydroxamic acid via an acid chloride intermediate in a facile high yielding reaction. The methyl ester groups of 3 were removed via base-catalysed hydrolysis to afford mal-\(p\)-Bel in high yield and excellent purity. The compound was characterized by elemental analysis, \(^1\)H NMR, \(^{13}\)C NMR and IR spectroscopy and mass spectrometry. NMR data is consistent with the proposed structure of mal-\(p\)-Bel. In the \(^1\)H NMR spectrum the carboxylic acid peaks are evident at 12.72 ppm integrating for two protons and the hydroxamate NH and OH signals are found either side of the sulphonamide NH (10.26 ppm) at 10.88 and 9.15 respectively. The aromatic protons for the di-substituted ring with the olefin/hydroxamic acid chain are found as two merged doublets at 7.69 and the second ring as doublets at 7.08 and 6.97 ppm. Finally the olefin protons are observed at 7.45 and 6.51 ppm respectively and the methyne and methylene protons at 3.47 and 2.90 ppm respectively. In the \(^{13}\)C NMR spectrum the carboxylic acid carbonyl carbon signals and hydroxamic acid carbonyl carbon signal are found at 170.13 and 162.01 ppm respectively. The 12 aromatic carbons are found in the range 120 to 140 ppm and the two olefin carbons at 136.49 and 122.21 ppm. The methyne and methylene carbons are found at 53.23 and 33.44 ppm respectively.
In the IR spectrum of mal-p-Bel the malonic acid v(C=O) is clearly evident at 1703 cm\(^{-1}\) and the symmetric and asymmetric hydroxamic acid v(C=O) are observed at 1656 and 1612 cm\(^{-1}\). The carboxylate v(O-H), hydroxamate v(N-H) and hydroxamate v(O-H) are tentatively assigned at 3491, 3243 and 2924 cm\(^{-1}\) respectively. ESI-MS in the negative mode assisted in identifying mal-p-Bel with a mass peak at 433.

Scheme 1 Synthesis of mal-p-Bel.

3.2 Synthesis of \(\text{cis-[Pt}^{II}(\text{NH}_3)_2\text{(mal-p-Bel}_{2\text{H}}))\) 5

The synthetic protocol for generating Pt-dicarboxylato complexes is well established.[5, 23] Reaction of the disodium salt of mal-p-Bel with \(\text{cis-[Pt}^{II}(\text{NH}_3)_2\text{(H}_2\text{O})_2\text{]}^{2+}\), generated from the reaction of iodoplatin and silver nitrate in the dark, afforded \(\text{cis-[Pt}^{II}(\text{NH}_3)_2\text{(mal-p-Bel}_{2\text{H}}))\), Scheme 2, in excellent yield (82\%) and purity. The complex was characterized by elemental analysis (\(^1\text{H NMR, }^{13}\text{C NMR, }^{195}\text{Pt NMR})\), IR spectroscopy and mass spectrometry.

Scheme 2 Synthesis of \(\text{cis-[Pt}^{II}(\text{NH}_3)_2\text{(mal-p-Bel}_{2\text{H}}))\).
Elemental analytical data for \( \text{cis-[Pt}^\text{II}(\text{NH}_3)_2(\text{mal-p-Bel})_{2\text{H}}]\) is consistent with the theoretical data for a diammine Pt(II) moiety bound to one mal-p-Bel\(_{2\text{H}}\) ligand. NMR data is consistent with the proposed structure of \( \text{cis-[Pt}^\text{II}(\text{NH}_3)_2(\text{mal-p-Bel})_{2\text{H}}]\). In the \(^1\text{H}\) NMR spectrum there is no evidence of the malonate protons, which was clearly evident at 12.72 ppm in the spectrum of mal-p-Bel, suggesting that the \( \text{cis-diammine Pt(II)} \) moiety is bound to mal-p-Bel \( \text{via} \) these dicarboxylate groups. The hydroxamate NH and OH are found either side of the sulphonamide NH (10.11 ppm) at 11.00 and 9.45 respectively. The aromatic protons for each di-substituted aromatic ring are found as two doublets at 7.84 and 7.76 and 7.19 and 7.08 ppm. The olefin protons are observed at 7.51 and 6.69 ppm and the ammine protons are observed as a broad peak at 4.44 ppm, integrating for six. Finally, the methyne and methylene protons are observed at 3.90 and 3.11 ppm respectively shifting upfield relative to those observed for the free ligand. In the \(^{13}\text{C}\) NMR spectrum the carboxylic acid carbonyl carbon signals and hydroxamic acid carbonyl carbon signal are found at 170.13 and 162.01 ppm respectively. The 12 aromatic carbons are, and as was the case for mal-p-Bel, found in the range \( \text{ca.} \) 120 to 140 ppm. The two olefin carbons are observed at 135.76 and 122.66 ppm. The methyne and methylene carbons are found at 59.94 and 34.16 ppm respectively. When comparing the \(^{13}\text{C}\) NMR of mal-p-Bel and \( \text{cis-[Pt}^\text{II}(\text{NH}_3)_2(\text{mal-p-Bel})_{2\text{H}}]\), where both are also referenced against TMS, significantly the two signals that shift are the carboxylic acid carbonyl carbon from 170.13 to 175.65 ppm and the methyne carbon from 53.23 to 59.94 ppm. These shifts support coordination of the the \( \text{cis-diammine Pt(II)} \) moiety to mal-p-Bel \( \text{via} \) the dicarboxylate groups. In the \(^{195}\text{Pt}\) NMR spectra the singlet observed at -1921 ppm correlates well with previously reported signals for PtN\(_2\)O\(_2\) type and in particular Pt ammine dicarboxylato complexes.[24]

IR spectroscopy is a particularly useful technique for characterisation of metal carboxylato complexes. On coordination of a carboxylic acid group to a metal it is well established that the corresponding carboxylate \( \nu(\text{C}=\text{O}) \) shifts to a lower wavenumber.[25] In the IR spectra of \( \text{cis-[Pt}^\text{II}(\text{NH}_3)_2(\text{mal-p-Bel})_{2\text{H}}]\) the carboxylate \( \nu(\text{C}=\text{O}) \) is not evident at 1703 cm\(^{-1}\) as was the case for mal-p-Bel. It is reasonable to suggest therefore that the carboxylate \( \nu(\text{C}=\text{O}) \) has shifted and is merged with the hydroxamic acid \( \nu(\text{C}=\text{O}) \) observed
at the lower 1656 cm\(^{-1}\). This observation strongly suggests that the cis-diammine Pt(II) moiety coordinates to mal-p-Bel via the dicarboxylate groups[25] and through the two deprotonated hydroxy groups to form a very stable six-membered chelate. ESI-MS in the positive mode assisted in identifying the complex with a mass of 660 and the correct isotopic pattern.

3.3 In vitro cytotoxicity

The in vitro anti-cancer potential of our test agents was determined using using two human-derived ovarian cancer cell lines; the parental cell line A2780 and the cisplatin resistant variant A2780 cisR. Dose-response curves were plotted (data not shown) and used to calculate IC\(_{50}\) values (Table 1). At 72 hr post-drug treatment, belinostat was found to be the most cytotoxic agent tested, with IC\(_{50}\) values of 0.6 and 0.7 µM against A2780 and A2780 cisR respectively. An IC\(_{50}\) value of 1.0 µM for belinostat against the A2780 cell line was previously determined using a CellTitre-Glo assay.[26] Mal-p-Bel was found to be considerably less cytotoxic than belinostat against A2780 cells (IC\(_{50}\) 45.8 µM) and A2780 cisR cells (IC\(_{50}\) 32.1 µM). Introduction of a malonate substituent onto the phenyl ring of belinostat clearly impacts on the cytotoxicity of the parent compound, belinostat. It is noteworthy though that mal-p-Bel exhibited greater cytotoxicity than its SAHA analogue, malSAHA, which was found to have IC\(_{50}\) values of greater than 200 µM against A2780 and A2780 cisR cells in previous studies.[5, 7]

Cisplatin, with IC\(_{50}\) values of 1.3 and 9.7 µM against A2780 and A2780 cisR cells respectively, was found to be slightly more cytotoxic than cis-[Pt\(^{II}\)(NH\(_3\))\(_2\)(mal-p-Bel\(_{2H}\))], which had IC\(_{50}\) values of 7.6 and 11.7 µM against A2780 and A2780 cisR cells respectively. cis-[Pt\(^{II}\)(NH\(_3\))\(_2\)(mal-p-Bel\(_{2H}\))] however possessed a superior resistance factor of 1.54 as compared to 7.46 for cisplatin.

As anticipated cis-[Pt\(^{II}\)(NH\(_3\))\(_2\)(mal-p-Bel\(_{2H}\))] exhibited enhanced cytotoxicity when compared to the previously reported Pt-malSAHA conjugate, cis-[Pt(NH\(_3\))\(_2\)(malSAHA\(_{2H}\))], which had IC\(_{50}\) values of 9.0 and 70 µM against A2780 and A2780 cisR cells respectively.[5] It is particularly noteworthy that cis-
[Pt\textsuperscript{II}(NH\textsubscript{3})\textsubscript{2}(mal-p-Bel\textsubscript{2H})] retains its cytotoxicity against the cisplatin resistant cell line, A2780 cisR, in contrast to Pt\textsubscript{II}alSAHA.[5]

In order to determine the potential cyto-selective nature of these test agents, their effect on cellular proliferation of a primary cell model, normal human dermal fibroblast (NHDF) cells, was examined. Only belinostat and \textit{cis}-[Pt\textsuperscript{II}(NH\textsubscript{3})\textsubscript{2}(mal-p-Bel\textsubscript{2H})] displayed noteworthy cyto-selectivity. Belinostat for example was 5 fold less cytotoxic towards the NHDF as compared to the A2780 cells. Similarly \textit{cis}-[Pt\textsuperscript{II}(NH\textsubscript{3})\textsubscript{2}(mal-p-Bel\textsubscript{2H})] was 4.5 times less toxic. However \textit{cis}-[Pt\textsuperscript{II}(NH\textsubscript{3})\textsubscript{2}(mal-p-Bel\textsubscript{2H})] was found to exhibit the lowest cytotoxicity (IC\textsubscript{50} 33.9 µM) against the NHDF cells and to be significantly less toxic when compared to both belinostat (IC\textsubscript{50} 3.0 µM) and cisplatin (IC\textsubscript{50} 2.4 µM).

\textbf{Table 1} Anti-proliferative effect of test agents against A2780 and A2780 cisR ovarian cell lines and NHDF human fibroblast primary cells following 72 hr continuous incubation with compound in the range 0-100 µM, using MTT assay. Results presented are representative of three independent experiments (Mean ± S.E.M., n=3).

<table>
<thead>
<tr>
<th>Cytotoxicity</th>
<th>Mean IC\textsubscript{50} (µM ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Line</strong></td>
<td>Cisplatin</td>
</tr>
<tr>
<td>A2780</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>A2780 cisR</td>
<td>9.7 ± 1.0**</td>
</tr>
<tr>
<td>NHDF</td>
<td>2.4 ± 0.6</td>
</tr>
</tbody>
</table>

(>, greater than). *p<0.05, **p<0.01 (Denotes significant difference between cell lines).

5. Conclusion
The potent HDACI, belinostat, was derivatised with the aid of molecular modelling to give a dicarboxylate analogue mal-p-Bel. It showed that introduction of a malonate group via a methylene linker para to the terminal benzene of p-Bel did not negatively affect the core binding of the hydroxamate group with the active site zinc cation. In fact, binding energies were improved with the addition of the malonate as described, likely due to new salt bridges forming between the malonate oxygen and the Lys202 of HDAC. A novel cis-diammine Pt complex of mal-p-Bel, cis-[Pt^{II}(NH_3)_2(mal-p-Bel-2H)], was successfully synthesised and characterised and is reported. An in vitro cytotoxicity study established that cis-[Pt^{II}(NH_3)_2(mal-p-Bel-2H)], though not as cytotoxic as belinostat nor cisplatin against A2780 and A2780 cisR ovarian cancer cells, possesses (i) considerable cytotoxicity against the reported ovarian cancer cells, (ii) enhanced cytotoxicity as compared to the previously reported cis-[Pt^{II}(NH_3)_2(malSAHA-2H)] and (iii) favourable cyto-selective properties as compared to cisplatin and belinostat. In addition, given cis-[Pt^{II}(NH_3)_2(mal-p-Bel-2H)] has the potential to act via two independent mechanisms, it is likely to have a greater capacity to circumvent resistance as compared to cisplatin for example. This study has therefore laid the foundations for an in depth mechanistic and proteomic study of cis-[Pt^{II}(NH_3)_2(mal-p-Bel-2H)].

References