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Anticancer Ru^{II} and Rh^{III} Piano-Stool Complexes that are Histone Deacetylase Inhibitors

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The first examples of Ru^{II} and Rh^{III} piano-stool complex histone deacetylase (HDAC) inhibitors are presented. The novel complexes have antiproliferative activity against H460 non-small-cell lung carcinoma cells that is comparable to the clinically used HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). Strong evidence for HDAC inhibition as a primary mechanism of action is provided. The complexes reported here represent an important step towards the design of highly active and selective HDAC inhibitors.

Historically the treatment of advanced or disseminated cancer has involved the systemic administration of cytotoxic compounds targeting nucleic acid replication or synthesis, many of which have been approved for clinical use since the 1960s.[1] Mechanistically these agents do not exclusively target cancer cells, and will also attack any rapidly proliferating cell type, commonly resulting in dose-limiting toxicity.[2] Over the past decade, increased understanding of the molecular basis of cancer has advanced cancer therapy into an era of "targeted molecular therapeutics".[3] This new class of targeted drugs exhibit a broad range of therapeutic mechanisms, including inhibition of extracellular growth receptors,[4] activation of cell death pathways,[5] retardation of cell motility,[6] kinase inhibition,[7] and toxin delivery,[8] to name a few. Subsequently, inhibition of enzymes associated with key regulatory pathways in cancer is an attractive alternative to targeting DNA.[9] In principle, "molecularly targeted" agents are highly selective agents against the growth and survival of tumour cells, whilst sparing normal cells.

The histone deacetylases (HDACs) are a class of enzymes recently shown to be suitable molecular targets for anticancer activity.[10] HDACs, working in tandem with histone acetylase transferases, control the extent of acetylation of ε-lysine residues in the tail of histone proteins[11] and several other cellular proteins, such as tubulin.[12] In terms of histones, deacetylation leads to a positively charged histone core, which interacts strongly with DNA, leading to a condensed chromatin structure. As a consequence, transcription of tumour-suppressor genes is repressed and cancer cell survival is promoted.[13] Consequently, HDAC inhibitors have received much attention as drug candidates, with suberoylanilide hydroxamic acid (SAHA, Figure 1) approved for clinical use against cutaneous T-cell lymphoma.[14] The hydroxamic acid group in SAHA binds to a Zn^{2+} ion located at the bottom of a hydrophobic cavity in the active site of HDAC enzymes.

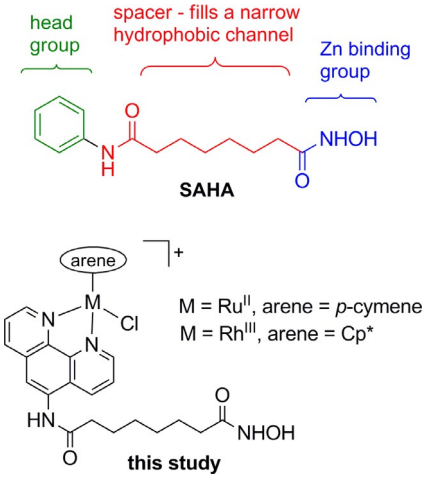

Figure 1. The HDAC inhibitor SAHA and the piano-stool complexes featured in this study. Cp* = pentamethylcyclopentadienyl.

It is known that the HDAC protein family is comprised of several sub-families demonstrating a wide range of roles across the cell, in addition to modulation of histone-regulated gene transcription.[15] Therefore there is significant interest in the development of HDAC inhibitors with the capability of selectively targeting a specific enzyme or sub-family,[16] with the objective of avoiding HDACs involved in normal physiological function and drug-induced toxicities.

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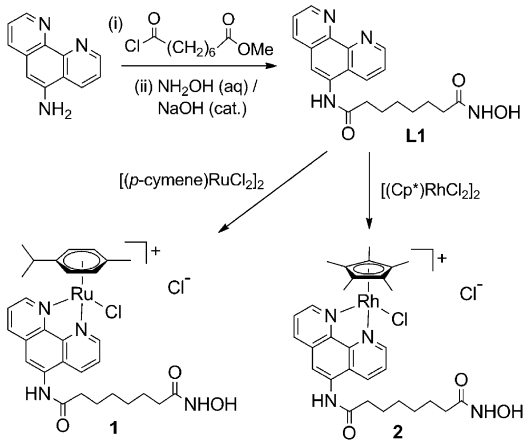
In terms of selectivity, although the enzymatic cavity is relatively comparable between HDACs, there is clear variability in the protein structure towards the entrance of the cavity. The phenyl headgroup of enzyme-bound SAHA sits in this region and offers scope for modification toward the development of HDAC-selective agents or more potent drugs through greater chemical affinity. In recent years, HDAC inhibitors have been developed in which the phenyl headgroup is replaced or functionalised with a metal complex. Examples include ferrocene-,[17] platinum-,[18] rhenium-[19] and ferrocifen-based[20] inhibitors. In each case the pharmacological effects are retained and, in some cases, improved cytotoxicity relative to SAHA was observed. Luminescent octahedral polypyridyl–metal complexes have also been developed.[21] The advantages of metal complexes over purely organic compounds in enzyme inhibition include: 3D metal geometries, allowing simultaneous access to multiple areas of the active site; exchangeable ligands, for in situ activation and potential binding to amino acid residues in the active site; simple and modular syntheses, allowing rapid determination of structure–activity relationships.

To be a successful selective headgroup, the metal complex would ideally have scope and functionality amenable to modification for optimising the interactions with the cavity entrance. One such class of metal-based compounds demonstrating these characteristics is the piano-stool complexes, comprising a d^6 low-spin metal core capped by a η^6 or η^5 aromatic ligand. Functionality is varied at the three remaining coordination sites of the pseudo-octahedral complexes, which are occupied by mono-, bi- or tri-dentate ligands. A large number of metal complexes based on this motif have been investigated for their anticancer activity,[22] with modification of each component leading to dramatic changes in activity. However, Ru^{II} and Rh^{III} piano-stool complexes have not previously been investigated as HDAC inhibitors.

Herein, we present the first examples of Ru^{II} and Rh^{III} piano-stool complexes that show effective HDAC inhibition and antiproliferative activity against H460 non-small-cell lung carcinoma cells. Our initial biological studies indicate that these complexes inhibit class I and II HDAC enzymes, but show no covalent binding to DNA.

We chose to use a substituted phenanthroline moiety, as this ligand is known to form stable chelates with the platinum group metals.[22i,23] Following a literature procedure,[21a] 1,10-phenanthrolin-5-amine and methyl 8-chloro-8-oxooctanoate were reacted to give a methyl ester intermediate. Without further purification, this intermediate was converted to ligand **L1** by the addition of hydroxylamine (50% aqueous solution) and catalytic base (Scheme 1). Upon neutralisation, **L1** precipitated and was collected by filtration and dried under high vacuum. Complexation of **L1** with selected metal dimers ($[(arene)MCl_2]_2$) was achieved using a 2:1 ratio of **L1**/metal dimer in anhydrous methanol. After removing the excess solvent, the crude product was purified by recrystallisation by dropping a concentrated CH_2Cl_2 solution into stirred Et_2O in a dry-ice/acetone bath (Scheme 1). Formation and purity of the complexes was confirmed using ^{1}H NMR spectroscopy, mass spectrometry and elemental analysis (see the Supporting Information for details).



Scheme 1. Preparation of piano-stool complexes of Ru^{II} (**1**) and Rh^{III} (**2**).

^{1}H NMR spectroscopy in [D$_6$]DMSO confirmed the presence of the intact hydroxamic acid, with broad resonances at 10.30 and 8.63 ppm corresponding to the hydroxamic acid OH and NH protons of complex **1**, respectively (10.38 and 8.63 ppm for complex **2**). These resonances are near identical to those of **L1**, confirming that chelation to Ru occurs only through the phenanthroline N donors. In contrast, the resonances for protons H2 and H9, adjacent to the phenanthroline N atoms, shift by almost 1 ppm upon complexation.

To assess the aqueous stability of the complexes, a solution of complex **1** in D_2O was monitored by ^{1}H NMR spectroscopy over the course of 96 h. After 1 h, an equilibrium was established between the chlorido complex **1** and the aqua species, in which the chlorido ligand has exchanged with D_2O. The chlorido/D_2O ratio is approximately 9:1 and remains unchanged over the course of 96 h (see the Supporting Information for full details). These results show that the complex is stable in aqueous solution and likely to remain intact as the chlorido species in biological media.

With the new complexes in hand, we first examined their ability to inhibit the proliferation of the H460 non-small-cell lung carcinoma cell line in vitro. Cells were exposed for 96 h to each new complex, the ligand **L1** and the known HDAC inhibitor, SAHA, at concentrations ranging from 0.01 to 200 μM. Cell survival was then determined by the MTT assay[24] and the IC_{50} (concentration of compound required to inhibit cell proliferation by 50%) was calculated from the resulting dose–response curve (see the Supporting Information for full details). The results (Table 1) show that the new complexes are able to effectively inhibit cell growth. The Ru^{II} complex with the capping *p*-cymene ligand (complex **1**) has an IC_{50} value of approximately 20 μM, which is comparable to that found in cytotoxicity studies of many other Ru piano-stool complexes,[22] but is 15-fold higher than for SAHA. However, the much lower cytotoxic efficacy (IC_{50}: 4 μM) demonstrated by the Rh^{III} complex, capped with a Cp* ligand (complex **2**) is comparable with the most active Rh^{III} piano-stool complexes reported to date.[25] The lower IC_{50} value of complex **2** and the fact it is approaching that of the clinically approved anticancer agent, SAHA (IC_{50}:

Table 1. IC_{50} values measured using the MTT assay (96 h) against the non-small-cell lung carcinoma H460 cell line, and are reported as the mean value from at least three experiments.

Compound	Arene–metal	IC_{50} [μM]
1	*p*-cymene–Ru^{II}	21 ± 6
2	Cp*–Rh^{III}	4.1 ± 0.4
L1	–	1.5 ± 0.2
SAHA	–	1.4 ± 0.2

1.4 μM), gives us encouragement that piano-stool complexes have the potential to act as HDAC inhibitors. Within experimental error the ligand **L1** has the same activity as SAHA.

To investigate whether these complexes act by HDAC inhibition, as proposed, we carried out enzyme inhibition assays, using a commercially available assay kit.[26] Fluorescence measurements were used to determine the extent of HDAC activity, with no fluorescence indicating complete HDAC inhibition. The known inhibitor SAHA, **L1** and each new complex were incubated at 0.1 and 1 μM with a nuclear extract source of HDACs, prior to the addition of an acetylated substrate. As a positive control, the assay was also run in the absence of any inhibitor. Results are presented as a percentage of HDAC activity, relative to the positive control (Table 2). For all compounds tested at

Table 2. HDAC activity in presence of potential inhibitors at 0.1 and 1 μM concentration, measured using a commercially available assay kit. Values are reported as percentage activity relative to a positive control (no inhibitor).

Inhibitor $conc^n$ [μM]	Control [%]	SAHA [%]	**1** [%]	**2** [%]	**L1** [%]
1	100	0.5	1.6	1.1	4.9
0.1	100	6.3	17.3	15.4	10.7

1 μM concentration, HDAC activity is low (< 5% activity), showing that these species are effective HDAC inhibitors. At 0.1 μM, HDAC activity is increased, but remains low, supportive of the inhibitory potency of these compounds. Although all tested compounds inhibit HDAC activity to the same order of magnitude, the extent of HDAC inhibition at the lower concentration follows the order SAHA > **L1** > **2** > **1**. This order mirrors the in vitro anticancer activity, which supports the hypothesis that HDAC inhibition is a putative mechanism of action of these species. Beyond this empirical observation, there are some interesting features within the results. Firstly, complex **2** showed a fourfold greater cytotoxic potency than complex **1**, but comparable HDAC inhibitory activity. This would suggest that the lower anticancer activity of the Ru^{II} complex is not entirely down to weaker HDAC inhibition. More likely, variation in processes such as cell uptake, localisation and egress of the compounds lead to the observed differences in cytotoxicity. A second observation from the data is that, despite being more active at 0.1 μM, at the higher concentration of 1 μM, the ligand **L1** leads to less enzyme inhibition than the complexes **1** and **2**. This might be due to aggregation of the planar aromatic **L1** at higher concentration,[27] leading to a reduction in compound available to bind to the enzyme, or might be due to lower solubility in the assay medium.

As a control, we measured the extent of HDAC inhibition by the known complex [(*p*-cymene)Ru(phen)Cl]Cl.[28] As expected, this complex shows no significant inhibition (100% HDAC activity at 1 μM complex), confirming that the hydroxamic acid moiety is essential for HDAC inhibition.

The HDAC assays clearly indicate that the new complexes are effective inhibitors of these enzymes. However, we wanted to determine whether this was the only mechanism of action that led to the observed cytotoxicity. Indeed, the majority of anticancer Ru^{II} piano-stool complexes are postulated to act through covalent binding to DNA. To test whether the complexes investigated herein interact with DNA, either through covalent modification or intercalation, DNA binding assays were performed. Firstly, to probe the ability of the complexes to covalently modify DNA, supercoiled pSG483 plasmid DNA was exposed to increasing concentrations of complex **2** and the resulting products were separated by agarose gel electrophoresis (Figure 2A). In comparison to a solvent only control (Figure 2A, lane 1), the migration of the supercoiled DNA is unaffected by complex **2**. As a comparison, the known DNA-binding complex cisplatin was examined under identical conditions (Figure 2B). As the concentration of cisplatin was increased, the compound formed covalent adducts with DNA that migrated more slowly, reaching a maximal shift at 2.5 μM. Above this concentration, the DNA signals became more disperse, likely indicating degradation of the DNA at higher concentrations of the compound. It is clear from this comparison that complex **2** does not covalently bind DNA.

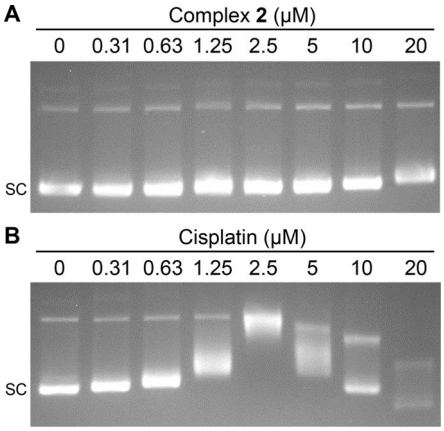


Figure 2. Covalent modification of DNA as determined by migration of substrate DNA during agarose gel electrophoresis. Supercoiled plasmid DNA was treated with increasing concentrations of complex **2** (A) and cisplatin (B). SC = supercoiled DNA.

Having confirmed that covalent binding to DNA is not favoured for complex **2**, we next explored the possibility of intercalation. Assays were run in which nicked pSG483 plasmid DNA was incubated with potential intercalators, then treated with DNA ligase (Figure 3). The ligase acts to re-seal the nicked DNA, trapping the current supercoiling state of the plasmid. In-

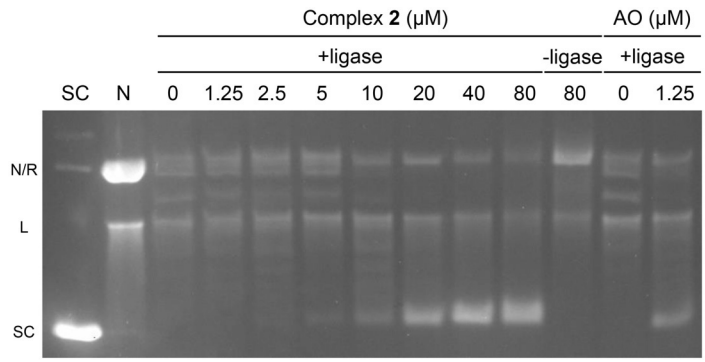


Figure 3. Intercalation of DNA demonstrated by the production of supercoiled DNA. Nicked plasmid DNA was treated with increasing concentrations of complex **2** and a positive control, acridine orange (AO). The supercoiled state was trapped by the addition of DNA ligase, where indicated. N/R = nicked/relaxed DNA, SC = supercoiled DNA, L = linear DNA.

tercalating compounds induce increased supercoiling within plasmid DNA, whereas non-intercalated nicked DNA treated with ligase will be sealed in a distribution of relaxed DNA topoisomers (Figure 3, lane 3). No intercalation was observed for concentrations of complex **2** below 20 μM, indicating that intercalation was not occurring at concentrations capable of causing HDAC inhibition or cytotoxicity. At higher doses, 20–80 μM of complex **2**, moderate intercalation could be observed. As a positive control, the known DNA intercalator acridine orange was tested at 1.25 μM, producing supercoiled DNA and demonstrating that in comparison, complex **2** does not intercalate DNA (Figure 3, lane 13).

From the assays carried out to determine one or more mechanism(s) of action, it is clear that HDAC inhibition is a potential therapeutic mechanism of anticancer activity in vitro. The results of our biological assays rule out covalent binding to DNA in a manner akin to cisplatin or many other Ru^{II} piano-stool complexes. Similarly, complex **2** does not intercalate with DNA at efficacious concentrations, as might be expected from a complex incorporating a planar aromatic ligand. Hence, we can be confident that a viable mechanism of anticancer activity of the new Ru^{II} and Rh^{III} complexes is through HDAC inhibition.

In conclusion, we have presented the first examples of Ru^{II} and Rh^{III} piano-stool complexes that inhibit HDAC enzymes, leading to growth inhibition of a lung carcinoma cell line. These complexes have comparable activity to the clinically approved inhibitor SAHA. The key advantage to using 3D piano-stool complexes for this application is the ease in which the structure of the metal complex can be varied. For example, the capping arene group or monodentate halide can be readily modulated to form 3D structures that might access new areas on the enzyme surface, leading to more efficient binding. This so-called "escape from flatland"[29] is much harder to envisage for the purely organic inhibitors, the synthesis of which would require longer and more challenging pathways. We are currently using computational modelling to aid in the design of more efficient piano-stool complex HDAC inhibitors.

Furthermore, whereas SAHA is a pan-HDAC inhibitor, the design of inhibitors that are selective towards a particular isoform is at the forefront of research in this area.[16] Piano-stool complexes, such as **2**, provide a platform from which selective HDAC inhibitors can be designed and synthesised with comparative ease. The use of selective inhibitors should provide more insight into the physiological roles of the HDAC isoforms and might reveal undiscovered functions of this important enzyme.

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Keywords: antitumour agents · bioorganometallic chemistry · histone deacetylase inhibitors · piano-stool complexes · ruthenium

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