A GENUINELY MULTIDISCIPLINARY JOURNAL

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Accepted Article

Title: Anticancer Ru(II) and Rh(III) Piano Stool Complex HDAC Inhibitors

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To be cited as: ChemPlusChem 10.1002/cplu.201600413

Link to VoR: http://dx.doi.org/10.1002/cplu.201600413

A Journal of

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Anticancer Ru(II) and Rh(III) Piano Stool Complex HDAC Inhibitors

Jasmine M. Cross,[a] Tim R. Blower,[b] Natalie Gallagher,[c] Jason H. Gill,[c] Kimberly L. Rockley,[c] and James W. Walton*[a]

Abstract: Piano stool metal complexes have been explored for several decades as potential anticancer agents. HDAC enzymes are excellent targets for such therapeutic activity. We present the first examples of Ru(II) and Rh(III) piano stool HDAC inhibitors. The novel complexes have anticancer activity comparable to the clinically used HDAC inhibitor 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] These new 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 growth and survival of tumor 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-lysines 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 are repressed and cancer cell survival is promoted.[13] Consequently, HDAC inhibitors have received much attention as drug candidates, with suberoyl 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 end of a hydrophobic cavity in the active site of HDAC enzymes.

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.

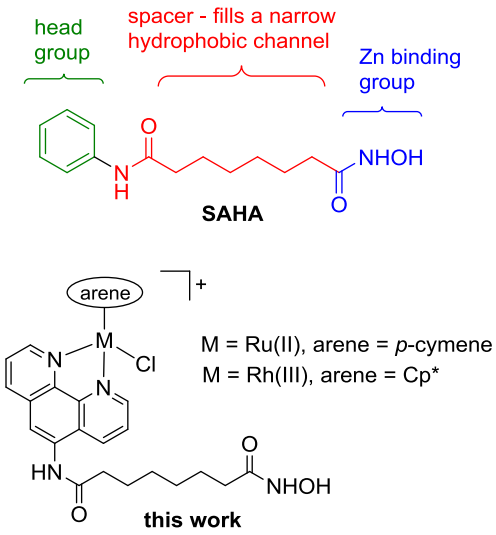

Figure 1. HDAC inhibitor SAHA and the piano stool complexes featured in this work.

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. In SAHA, the phenyl head group sits in this region and offers scope for modification toward development of HDAC-selective agents or more potent drugs through greater chemical affinity. Over the past few years, HDAC inhibitors have been developed in which the phenyl head group is replaced or functionalised with a metal complex. Examples include ferrocene-,[17] platinum-,[18] rhenium-[19] and ferricofen-based[20] inhibitors. In each case the pharmacological effects are retained and, in some cases, improved cytotoxicity relative to SAHA is observed. Luminescent octahedral polypyridyl metal complexes have also been developed.[21] The advantages of metal complexes over purely organic compounds in enzyme inhibition include: 3-dimensional metal geometries, allowing simultaneous access to multiple areas of the active site; exchangeable ligands, for *in situ* activation and potentially binding to amino acid residues in the

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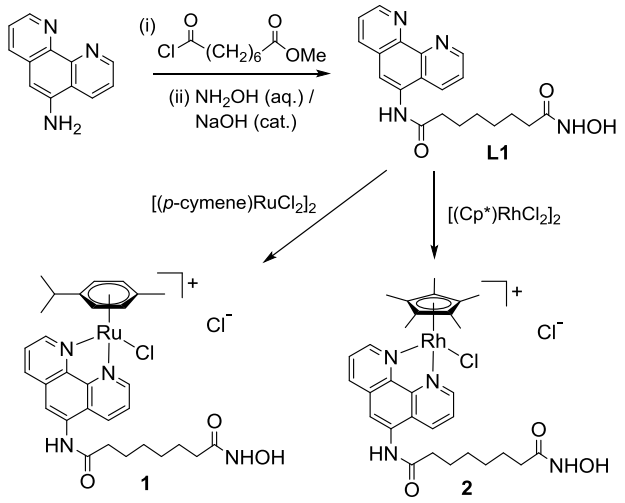
active site; simple and modular syntheses, allowing rapid determination of structure activity relationships.

In order to be a successful selective headgroup, the ideal metal complex would need to have scope and functionality amenable to modification for exploration and 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 an η^6 or η^5 aromatic ligand. Amenable functionality is obtained by 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.



Scheme 1.

We present the first example of Ru(II) and Rh(III) piano stool complexes which show effective HDAC inhibition and antiproliferate 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 pursue a substituted phenanthroline moiety, as this ligand is known to form stable chelates with the platinum group metals.[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. 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 ration of **L1** : metal dimer in anhydrous methanol. After removing the excess solvent, the crude product was purified by recrystallisation, by dropping a concentration 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, mass spectrometry and elemental analysis (see ESI for details†). ^{1}H-NMR 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 phenanthroline N, shift by almost 1 ppm upon complexation.

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

Table 1. IC_{50} values measured using the MTT assay (96 h) against the non-small cell lung carcinoma H460 cell line. Entries are the mean value for data 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

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 10 nM 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%) calculated from the resulting dose response curve (see ESI 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 IC_{50} value around 20 μM, which is comparable to cytotoxicity studies of many other Ru piano stool complexes reported,[22] but is 15-fold higher than 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}: 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.

Table 2. HDAC activity in presence of potential inhibitors at 100 nM and 1 μM concentration, measured using commercially available assay kit. Values are reported as percentage activity relative to a positive control (no inhibitor).

	Control	SAHA	**1**	**2**	**L1**
1 μM	100%	0.5%	1.6%	1.1%	4.9%
100 nM	100%	6.3%	17.3%	15.4%	10.7%

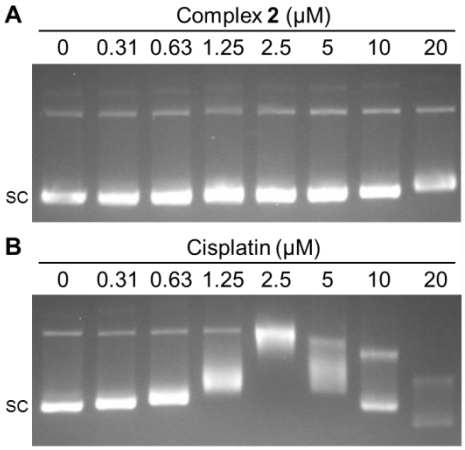


Figure 2. Covalent modification of DNA as determined by migration of substrate DNA during agarose gel electrophoresis. Supercoiled plasmid DNA was treated with increasing concentrations of (A) complex **2** and (B) cisplatin. A solvent-only control was used for 0 μM of each compound. SC, supercoiled DNA.

To investigate whether these complexes act via HDAC inhibition, as proposed, we carried out enzyme inhibition assays, using a commercially available assay kit.[26] Fluorescence measurements are 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 1 μM and 100 nM with the 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 tested compounds, at 1 μM concentration, HDAC activity is very low (< 5% activity), showing that these species are effective HDAC inhibitors. At 100 nM, HDAC activity is increased, but remains low, supportive of inhibitory potency. While all tested compounds inhibit HDAC activity to the same order of magnitude, the extent of HDAC inhibition 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 four-fold 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 poorer HDAC inhibition. More likely, variation in processes such as cell uptake, localisation and egress lead to the observed differences in cytotoxicity. A second observation from the data is that, despite being more active at 100 nM, at the higher concentration of 1 μM, the ligand **L1** leads to less enzyme inhibition than the complexes **1** and **2**. This may be due to some aggregation of the planar aromatic **L1** at higher concentration,[27] leading to a reduction in compound available to bind to the enzyme, or may 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 leads 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 (lane 1), the migration of the supercoiled DNA band 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 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 high concentrations of the compound. It is clear from this comparison that complex **2** does not covalently bind 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. Intercalative 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 (lane 3). No intercalation was observed in 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 (AO) was tested at 1.25 μM, producing supercoiled DNA at this low concentration and demonstrating that in comparison, complex **2** does not intercalate DNA (lane 13).

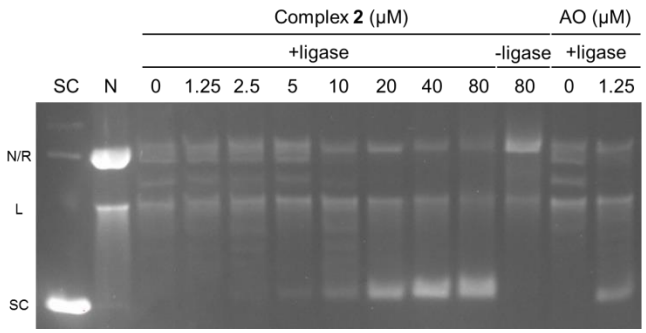


Figure 3. Intercalation of DNA demonstrated by the production of supercoiled DNA. Nicked plasmid DNA was treated with increasing concentrations of complex **2** and a positive control, acridine orange (AO). The supercoiled state was trapped by the addition of DNA ligase where indicated. A solvent-only control was used for 0 μM of each compound. N/R, nicked/relaxed DNA, SC, supercoiled DNA, L, linear DNA.

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*. 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 therapeutic 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 example of Ru(II) and Rh(III) piano stool complexes that inhibit the 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 3-dimensional 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 3-dimensional structures that can 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, whose formation would require longer and more challenging synthetic pathways. We are currently using computational modelling to aid in the design of more efficient piano stool complex HDAC inhibitors.

Furthermore, while 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. Selective inhibitors will provide more insight into the physiological roles of the HDAC isoforms and no doubt reveal undiscovered functions of this important enzyme.

Experimental Section

See Supporting Information for experimental details on Synthetic Procedures; MTT Assay Procedure; Enzyme Inhibition Assay and DNA Binding Experiments.

Acknowledgements

We thank Durham University and the EPSRC for funding.

Keywords: Ruthenium Anticancer Agents • HDAC Inhibitors • Piano Stool Complexes • Bioorganometallic Chemistry • Bioinorganic Chemistry

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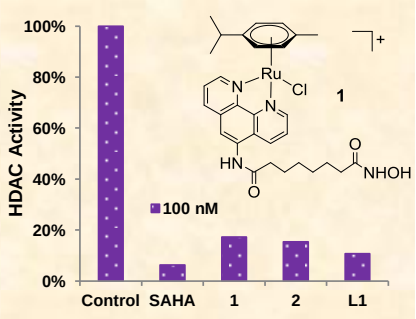
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

We present the first examples of Ru(II) and Rh(III) piano stool HDAC inhibitors. The novel complexes have anticancer activity comparable to the clinically used HDAC inhibitor SAHA. Strong evidence for HDAC inhibition as a primary mechanism of action is provided, but covalent binding to DNA is ruled out.



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Anticancer Ru(II) and Rh(III) Piano Stool Complex HDAC Inhibitors

Layout 2:

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