Multi-functional Copper(II) Drug Candidates as Potential Anti-Cancer Agents

A thesis submitted to the Royal College of Surgeons in Ireland for the degree of Doctor of Philosophy

By

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<th>Definition</th>
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<tbody>
<tr>
<td>Phen</td>
<td>1,10-Phenanthroline</td>
</tr>
<tr>
<td>Bipy</td>
<td>Bipyridine</td>
</tr>
<tr>
<td>Phendio</td>
<td>1,10-Phenanthroline 5,6-dione</td>
</tr>
<tr>
<td>DPQ</td>
<td>Dipyrido[3,2-d:2',3'-f]quinoxaline</td>
</tr>
<tr>
<td>DPPZ</td>
<td>Dipyrido[3,2-a:2',3'-c]phenazine</td>
</tr>
<tr>
<td>DPPN</td>
<td>Benzo[i]dipyrido[3,2-a:2',3'-c]phenazine</td>
</tr>
<tr>
<td>tmp</td>
<td>3,4,7,8-tetramethyl-1,10-phenanthroline</td>
</tr>
<tr>
<td>dmdppz</td>
<td>11,12-dimethylidipyrido[3,2-a:2'3'-c]phenazine</td>
</tr>
<tr>
<td>Cu-Phen</td>
<td>[Cu(Phen)$_2$]$^{2+}$</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>Superoxide radicals</td>
</tr>
<tr>
<td>$^\cdot$OH</td>
<td>Hydroxyl radicals</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>$^1O_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>IARC</td>
<td>International agency for research on cancer</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>cis[Pt(NH$_3$)$_2$(Cl)$_2$]</td>
</tr>
<tr>
<td>US FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>CTR1</td>
<td>Copper transporter 1</td>
</tr>
<tr>
<td>CBDC</td>
<td>1,1-cyclobutanedicarboxylate</td>
</tr>
<tr>
<td>DACH</td>
<td>1R,2R-diaminocyclohexane</td>
</tr>
<tr>
<td>NCI</td>
<td>National cancer institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>5-FU/LV</td>
<td>5-fluoroacil / leucovorin</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
</tbody>
</table>
pKw  Water ionisation constant
KCl  Potassium chloride
logβ  Stability constant
DMSO  Dimethylsulfoxide
PBS  Phosphate buffered saline
SA  Staphylococcus aureus
MRSA  Methicillin resistant *Staphylococcus aureus*
SE  *Staphylococcus epidermidis*
EF  *Enterococcus* sp.
EC  *Escherichia coli*
KP  *Klebsiella pneumonia*
PA  *Pseudomonas aeruginosa*
CA  *Candida albicans*
CK  *Candida krusei*
CT  *Candida tropicalis*
CG  *Candida glabrata*
CL  *Candida lusitaniae*
TA  *Trichosporon asahii*
AF  *Aspergillus fumigatus*
AC  *Aspergillus corymbifera*
TM  *Trichophyton mentagrophytes*
IC<sub>50</sub>  Inhibitory concentration 50%
µM  Micromolar
ELISA  Enzyme linked immunosorbent assay
Poly GC  Poly[d(G-C)<sub>2</sub>]
Poly AT  Poly[d(A-T)<sub>2</sub>]
stDNA  Salmon testes DNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctDNA</td>
<td>Calf thymus DNA</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>TH$_2$</td>
<td>4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt</td>
</tr>
<tr>
<td>SBW</td>
<td>Spectral bandwidth</td>
</tr>
<tr>
<td>Q</td>
<td>Quenching affinity</td>
</tr>
<tr>
<td>T$_M$</td>
<td>Thermal melting</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>8-Oxo-2'-deoxyguanosine</td>
</tr>
</tbody>
</table>
IV. Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed____________________________________

Student Number________________________________

Date__________________________________________
V. Acknowledgements

I would like to thank my primary PhD supervisor, Prof. Celine Marmion and co-supervisor Dr. Andrew Kellett for their kind wisdom and guidance throughout the course of these last three years. Both of you are remarkable scientists and it was a pleasure being your doctoral student in this early stage of my research career. Both of you have mentored me in this multi-disciplinary and multi-institutional research and given me an insight into critical analysis which will ultimately make me a better researcher. I’m sure I’ve given you numerous headaches over the past 3 years and hopefully I can deliver what you expected. I am forever grateful to Prof. Niamh Moran who gave me a second chance in life – particularly my research career - if it wasn’t for her I would not be in the position I am today.

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To my family, (and the better siblings) Niamh and Texas-Niall I appreciate the loving support and guidance you have given me over my PhD research and my life, I would also like to thank my extended family for their support during my doctoral research. Last but not least I dedicate this thesis to my parents, Hugh and Philomena.
VI. Honours

Peer Reviewed Conference Abstract Publications

2013


POSTER - Mc Givern, T.J.P and Marmion, C.J. Novel Multifunctional Mono- and Hetero-Nuclear Complexes of Histone Deacetylase Inhibitors as
Potential Anti-Cancer Therapeutics. *International Conference for Bio-Inorganic Chemistry 16 (ICBIC 16), Grenoble, France, 22-26th July 2013.*


2014


POSTER - Mc Givern, T.J.P; Kellett, A and Marmion, C.J. Development of Multi-Functional Cu(Phen) Metallonucleases as Potential Anti-Cancer...
Therapeutics. *Irish Universities Chemistry Research Colloquium, National University of Ireland Galway, 19th – 20th June 2014.*


**2015**

POSTER - **Mc Givern, T.J.P.; Kellett, A and Marmion, C.J.** Multi-Functional Redox Active Cu$^{II}$ Drug Conjugates. *RCSI Research Day 2014, Royal College of Surgeons in Ireland, 13 March 2015.*


VII. Abstract

The widespread clinical use of classical platinum drugs is hampered by a number of issues such as dose-limiting toxic side effects and intrinsic and/or acquired resistance in cancerous cells. The search for metallodrugs which (i) selectively target cancerous cells and thus have a more favourable toxicity profile to Pt(II) drugs and/or (ii) have a mechanism of action different to classical Pt drugs and thus potentially overcoming resistance issues, continues to be the subject of intense investigation in the field of medicinal bioinorganic chemistry. Copper complexes offer an attractive alternative owing to their redox active properties and DNA binding and chemical nuclease activities. Already a large array of Cu complexes have been developed as anti-cancer agents with two already undergoing clinical trials.

In parallel, the design, synthesis and evaluation of enzyme inhibitors rationally chosen for their potential in suppressing tumour cell proliferation have also shown promise, one such example being histone deacetylase (HDAC) inhibitors. The clinically approved HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), for example, not only possesses potent anti-cancer activity but also demonstrates selectivity towards tumour cells over normal cells. It is well tolerated by patients at doses which induce a potent anti-cancer effect.

There have been no reports to date of Cu(II) complexes in which the Cu(II) ion is coordinated to both (i) a HDAC inhibitor and (ii) various designer DNA intercalating ligands. We sought to fill this void by generating innovative multi-functional Cu(II) complexes possessing both HDAC inhibitory and DNA cleavage properties.
Herein we describe the rational design, synthesis and characterisation of a novel series of copper(II) chemotypes of structural formula \( X \text{-Cu-Y} \) where \( X \) is the clinically used HDAC inhibitor SAHA and \( Y \) is a designer intercalating ligand – selected from Bipy, Phen, Phendio, DPQ or DPPZ (where Phen is 1,10-phenanthroline, Bipy is bipyridine, Phendio is 1,10-phenanthroline-5,6-dione, DPQ is dipyrido[3,2-f:2',3'-h]quinoxaline and DPPZ is dipyrido[3,2-a:2',3'-c]phenazine), Figure 1. All complexes were generated via a simple one-pot reaction in good yield and excellent purity. Speciation studies were conducted in order to provide insight into the stability and behaviour of these systems in solution over a wide pH range. Stability constants of these Cu(II) chemotypes in a DMSO:H\(_2\)O solvent mixture are also presented.

![Chemical structures](image)

**Figure 1** Multifunctional Cu(II) complexes synthesized

In order to assess the potential of these chemotypes to act as anti-cancer agents, their *in vitro* cytotoxic activity was assessed across a panel of cancerous cell lines as well as their ability to inhibit HDAC function. Their anti-bacterial and anti-fungal activities were also assessed given that many Cu(II) complexes possess these properties. These complexes have significant *in vitro* cytotoxic activity across a panel of cancerous, fungal and bacterial cell lines. Through a structure-activity relationship study we have identified three lead drug candidates which were found to have enhanced activity against both prostate (DU145) and cisplatin resistant ovarian (SK-OV-3) cancerous cell lines. All complexes were capable of inhibiting HDAC function in SK-OV-3 cells within a shorter time interval compared to the clinical agent, SAHA, at isotoxic concentrations. We also found that Cu(II) complexes were active against Gram positive bacteria, and the Cu-SAHA-DPPZ chemotype showed selective activity against a non-filamentous fungal strain.
We have also found that by changing the DNA intercalating ligand, we can optimise the binding affinity of these chemotypes towards calf thymus DNA (ctDNA). Our results suggest that these chemotypes preferentially stabilise G-C rich regions of DNA and can intercalate at both the major and minor DNA grooves. Furthermore, potent oxidative DNA cleavage activity of the Cu(II) chemotypes was observed with activity on par with the well-cited chemical nuclease agent, [Cu(Phen)$_2$]$^{2+}$. Scavengers were also employed to identify the main ROS species produced during the reaction of the Cu(II) chemotypes with DNA. O$_2$•$^{-}$ and ·OH were found to be the predominant species with H$_2$O$_2$ to a lesser extent, playing a role.
For my Parents,

Phil & Hughie
Chapter 1 - General Introduction
1.1 Cancer
Cancer, as a disease, remains a persistent problem within our society, regardless of age, race or cultural background, with reported diagnosis of several cancers on the rise. The International Agency for Research on Cancer (IARC), a Division of the World Health Organisation, reported in its GLOBOCAN 2012\[1\] database that there were 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 compared to 12.7 million new cases and 7.6 million cancer deaths in 2008.\[1\] The IARC predicts that new cancer cases will rise to 19.3 million by 2025.\[1\] The highest incidence of cancer deaths were found to be breast, prostate and lung cancer.\[1\] In Ireland, 30.5% of recorded deaths in 2011 were cancer-related.\[2\] Ireland was recently ranked 3\textsuperscript{rd} in the European Union for cancer deaths.\[2\]

Although research into cancer has yielded a number of important findings to better understand the disease,\[3\] the development of novel therapeutics to overcome mechanisms of cancer cell resistance towards current therapies continues to be a focal point in cancer research (Scheme 1).

Scheme 1 An overview of drug design efforts.
1.2 Brief history of metal complexes as therapeutic agents

Medicinal inorganic chemistry is a thriving research field which is concerned with the design, synthesis and biological evaluation of innovative metal complexes as drug candidates. The advancement of this field was undoubtedly due in part to the enormous clinical success of the platinum (II) drug cisplatin as an anti-cancer agent, and its second generation analogues carboplatin and oxaliplatin (section 1.3).

Metals, and in particular transition metals, offer a number of potential advantages over organic-based drugs in that they possess a wide range of coordination numbers and geometries and thus greater structural diversity.[4] In addition, some metals are redox active, a property which could be potentially exploited given the different redox environments within the body. The thermodynamic and kinetic properties of metal complexes can also be fine-tuned through ligand substitution.[4] Metal complexes consist of a cationic metal centre bonded to molecules (or ligands) via coordinate or dative bonds. The geometrical arrangement of a complex is dependent on not only the number and type of ligands bonded to the central metal ion but also the coordination preference of the metal ion which can vary depending on its oxidation state. Geometries include: trigonal planar, tetrahedral, trigonal bipyramidal, octahedral, pyramidal, square planar, square pyramidal and trigonal prismatic (Figure 2).

![Figure 2 Graphical representation of coordination geometries adopted by metal complexes.](image-url)
The use of metals in medicine is not a new phenomenon. Ancient Greeks used copper(II) sulfate to sterilise drinking water in 3000 BC. Gold-based medicines have been used in China and Arabia as far back as 2500 BC and even to this day gold is used in dentistry for both fillings and crowns. Mercury has been used in medicine for almost 200 years. Mercury(I) chloride (HgCl), for example, was used predominantly in the 16th century as a diuretic and laxative in Europe and later found use as a treatment for syphilis, often poisoning the patient. It wasn’t until the 19th century that HgCl gained popularity as ‘blue mass’ commonly sold as a tonic or in tablet form for the treatment of ailments including tuberculosis, childbirth pains, constipation, toothache and parasitic infections. Today, the use of mercury complexes has ceased due to their toxic side effects. They are still however found in traditional Chinese, Tibetan and Ayurvedic medicines.

Arsenic, throughout the years, has been prescribed for a range of diseases such as rheumatoid arthritis, malaria, tuberculosis and diabetes. Although arsenic is a known poison it was used by a number of predominant physicians including Hippocrates (460 to 377 BC) as escharotics in the form of orpiment (As$_2$S$_3$) and realgar (As$_2$S$_2$). Arsphenamine (Salvarsan), an arsenic-containing compound, was the first reported effective treatment for syphilis. Despite the use of metals in medicine over the centuries, it wasn’t until the 1960s, when the anti-cancer properties of cisplatin were serendipitously discovered, that the scientific community began to conduct widespread research into the use of metal complexes as therapeutic agents; an era that later became known as a ‘renaissance of bioinorganic chemistry’ and also when the term ‘biocoordination chemistry’ was coined.
1.3 Pt and its role in the clinic

1.3.1 Cisplatin as an anti-cancer agent

Cisplatin – cis-[Pt(NH$_3$)$_2$(Cl)$_2$] (US trade name Platinol) (Figure 3, panel B) was first synthesized by the Italian chemist Michele Peyrone in 1844[5] and, for a long period of time, was known as Peyrone’s chloride. The chemical structure of cisplatin was reported by Alfred Werner in 1893.[6] It was not however until 1965 when Barnett Rosenberg and colleagues were investigating the effects of electrical fields on bacterial growth that the antibacterial properties of cisplatin were serendipitously discovered; Pt species which formed in solution were found to inhibit cell division of Escherichia coli (E. coli) cells but not cell growth.[7] Rosenberg realised that the Pt compounds which inhibited bacterial cell division but promoted cell growth might stop cell division in cancer cells (in which cell growth is uncontrollably rapid). A number of Pt species were isolated and tested for anti-cancer activity with the square planar Pt(II) complex cis-[Pt(NH$_3$)$_2$(Cl)$_2$] or cisplatin identified as being the most potent. Cisplatin was later found to be highly effective at regressing sarcomas in mice.[8] Further testing on other tumour cell lines ultimately led to the clinical approval of cisplatin by the United States Food and Drug administration (US FDA) in 1978 for the treatment of testicular and ovarian cancers. Since its clinical introduction, cisplatin has found use as a treatment for a wide range of tumours including bladder, ovarian, cervical, non-small cell lung cancer, squamous cell carcinoma, mesothelioma and testicular cancer usually as combination regimes.[9]

The mechanism of action of cisplatin is not fully understood. Cisplatin first enters cancerous cells by either passive diffusion and/or active uptake. Studies have shown that the copper transporter protein CTR1 plays a major role with respect to its initial influx into cancerous cells.[10] Cisplatin is essentially a prodrug where, upon cell entry, it is activated by hydrolysis with replacement of one or both chlorido ligands in cisplatin with water generating either its mono-or-bis aqua species (cis-[Pt(NH$_3$)$_2$Cl(OH)$_2$])$^+$ and cis-
[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$] respectively. It is these species which have the capacity to form various inter- or intra-strand crosslinks with deoxyribonucleic acid (DNA) (Figure 3, panel A). Formation of Pt-DNA adducts blocks the binding of various transcription/translation factors ultimately inducing apoptotic cell death. The widespread clinical use of cisplatin unfortunately is hindered due to a number of drawbacks. Cisplatin causes serious side effects such as myelosuppression, nausea, bone marrow suppression and nephrotoxicity.$^{[11]}$ Resistance in cancerous cells, both intrinsic and acquired, is also a significant drawback.$^{[12]}$ The second generation analogues of cisplatin, namely carboplatin and oxaliplatin, were developed in order to address these shortcomings.

Figure 3 Clinically-used Pt(II) drugs: Panel A: X-ray crystal structure of DNA (shown in red) containing a cisplatin crosslink (cisplatin shown in white/grey).$^{[13]}$ Panel B: Chemical structures of the Pt(II) drugs cisplatin, carboplatin and oxaliplatin.
1.3.1 Second generation Pt(II) drugs – carboplatin and oxaliplatin

*Carboplatin*

The toxicity of cisplatin is thought to be related to the lability of the chlorido ligands resulting in cisplatin having a relatively short half-life. Cisplatin is quite promiscuous in that it reacts readily and indiscriminately in the body. Cisplatin is susceptible to attack by, for example, sulphur-containing biomolecules and the resulting complexes are thought to give rise to the toxicity profile associated with its use. Replacing the chlorido ligands with an O,O'-bidentate chelating agent 1,1-cyclobutanedicarboxylate (CBDC) results in the formation of the structurally similar but significantly more stable carboplatin (Figure 3, panel B) or diammine[1,1-cyclobutanedicarboxylato(2-)-O,O']platinum(II) (US trade name Paraplatin). Carboplatin, although having a similar mechanism of action to cisplatin,\textsuperscript{[14]} because of its longer half-life, has a significantly reduced toxicity profile. Carboplatin is essentially devoid of nephrotoxicity and less toxic to the GI tract and also less neurotoxic; however thrombocytopenia is dose-limiting.\textsuperscript{[11b]} A higher dose of carboplatin (~20-40 fold increase) is however required to have an equal DNA binding effect as compared to cisplatin.\textsuperscript{[15]} Numerous randomized clinical trials of carboplatin *versus* cisplatin treatment have reported essentially equivalent survival rates in patients with ovarian cancer.\textsuperscript{[16]} Due to its more favourable toxicity profile, carboplatin is now considered a first line treatment in patients with high-risk early stage ovarian cancer.\textsuperscript{[17]}

*Oxaliplatin*

Oxaliplatin, another square planar Pt(II) complex, adopts a structure more similar to carboplatin. The two chlorido ligands have been replaced with a bidentate oxalato leaving group while the ammine groups have been replaced with a 1R,2R-diaminocyclohexane (DACH) bidentate ligand (Figure 3, panel B). The mechanism of action of oxaliplatin differs somewhat to that of both cisplatin and carboplatin; while the Pt binds DNA nucleobases, the
presence of the bulky DACH moiety also interacts with the DNA backbone furnishing oxaliplatin with a different biological profile to that of cisplatin and carboplatin. Although oxaliplatin forms fewer DNA adducts than cisplatin,\textsuperscript{18} the adducts appear to be more effective at blocking DNA replication as compared to carboplatin and cisplatin.\textsuperscript{19} Oxaliplatin, when tested in the National Cancer Institute (NCI) 60-cell human tumour panel, differed in sensitivity to cisplatin\textsuperscript{20} while retaining activity against some cancerous cells which possess acquired resistance to cisplatin.\textsuperscript{19} Oxaliplatin (US trade name Eloxatin) was approved by the US FDA in 2002 for the treatment of colorectal cancer in combination with 5-fluorouracil/leucovorin (5-FU/LV).\textsuperscript{21}

Despite the aforementioned shortcomings of classical Pt drugs, they have played a pivotal role in modern clinical oncology. In fact, \textasciitilde50\% of all cancer treatment regimens are Pt-based\textsuperscript{22}. With DNA as the primary target of classical Pt drugs, it is not surprising that there has been a surge in interest into DNA as a potential target for other metallodrug molecules in recent years.

1.4 DNA as a metallodrug target

1.4.1 DNA structure and function

DNA is a large polymer consisting of repeating units of deoxyribose, phosphate and a nucleotide base (Figure 4, Panel A). DNA is an essential component of all living organisms as DNA contains the genetic information inside living cells.
Figure 4 DNA structure: Each DNA nucleotide is composed of a phosphate group, a molecule of deoxyribose sugar and a nitrogen base (G, C, A, T).

DNA is essentially a large polymer consisting of two complementary chains held together by hydrogen bonding (H-bonding). At its core, DNA is composed of a series of monomers referred to as nucleotides. Each nucleotide is composed of a purine (Adenine – A; Guanine – G) or pyrimidine (Cytosine – C; Thymine – T) base, a deoxyribose sugar and phosphate group (Figure 4). Nucleotides are held together by covalent bonds between the oxygen atom of the phosphate and the 3’ carbon of the deoxyribose sugars, this forms a “backbone” of alternating sugar-phosphate moieties. The alternation of these groups gives rise to the direction of the DNA backbone: the 3’ end of DNA always contains a deoxyribose sugar while the 5’ end always contains a phosphate group. The DNA polynucleotide strands are complementary to each other where base pairs adopt Chargaff’s rule i.e. G always forms H-bonds with C and A always forms H-bonds with T. [23]
To minimize energy and maximise base pair stacking efficiency, the two DNA backbones wind around each other to form an elegant double helix. In the most common DNA structure, B-DNA, the helix is wound in a right hand fashion with about 10-10.5 base pairs per turn, with a centre-to-centre distance of 3.4 nm. In the double helix, the coiling of the DNA strand creates two distinct regions, a ‘major’ groove of 22 Angstroms (Å) in width and another smaller ‘minor’ groove 12 Å wide (Figure 5). These grooves are formed due to H-bonding between complementary DNA base pairs causing the deoxyribose sugar groups to project at a 120° angle from each other.

![DNA structure](image)

**Figure 5** DNA structure: Structure of B-DNA showing minor and major grooves. Adapted from Microsoft Office® clipart.

There are two main forces of attraction holding the B-DNA double helix together; H-bonding interactions between complementary base pairs inside the double helix and van der Waals’ base-stacking interactions between parallel nucleotides.\[24\] Specifically, H-bonding occurs between complementary base pairs (Figure 4, Panel C). G and C base pairs form three H-bonds whereas A and T base pairs form two H-bonds. Because of this additional H-bonding interaction between GC base pairs, DNA strands with a high GC content are more stable compared to those with a high AT content.\[25\]
1.4.2 Metal ion binding sites on DNA

DNA presents an interesting target for metallodrugs. Metal ions can interact either directly with DNA, for example, through interactions with nucleic acids such as those involving cisplatin. In contrast, metal complexes can interact indirectly with DNA through non-covalent interactions, for example, groove binding or intercalation.\[26\]

1.4.3 Non-covalent DNA binding

A number of clinically used agents such as anti-tumour, antiprotozoal, antiviral and anti-bacterial drugs interact with DNA via non-covalent interactions. Compounds which exert a DNA targeting effect with high specificity can influence gene expression and affect cell proliferation and differentiation. Novel metal complexes as anti-cancer agents have been developed with this in mind.

*Groove binding:* A groove binding agent is a compound which interacts at either the DNA major or minor groove. Minor groove binders can be broken into two classes: (i) a binder which induces permanent damage through covalent interactions with nucleophilic components of the minor groove (e.g. N3 of adenine or the 2-amino group of guanine); or (ii) compounds which reversibly inhibit DNA function via non-covalent interactions (e.g. H-bonding interactions, Van der Waals’ interactions).\[27\] Minor groove binding agents tend to have similar structural properties i.e. they tend to have an annular curvature with multiple aromatic rings which match the curvature of the DNA minor groove. These compounds also tend to be cationic resulting in a high affinity for the negative potential in the groove. Binding of these agents in the minor groove tends to result in little or no distortion of the DNA backbone concomitantly stabilising the B-DNA structure.\[27b\]

*Intercalation:* Intercalation, in contrast, involves the insertion of typically a planar aromatic molecule, between two DNA base pairs which causes
concomitant widening and lengthening of the DNA strand. This widening causes the base pairs to separate, creating an opening of about $3.4 \text{ Å}$. This opening induces local structural changes to the DNA strand such as lengthening or twisting of the DNA base pairs.

1.4.4 Classic groove binding and intercalating agents
The compounds Hoechst 33258 and netropsin (Figure 6, Panel B) are known DNA minor groove binding agents. Both bind at A-T rich regions of DNA. Netropsin binds with its three amide groups orientated towards the floor of the minor groove. Hoechst 33258 belongs to the bis-benzimide family of compounds. Hoechst 33258, when bound to the DNA minor groove, has fluorescent properties. Due to these fluorescent properties, Hoechst 33258 has also found use as a DNA stain. Hoechst 33258 also possess cytotoxic properties.

Ethidium bromide (EtBr) and Actinomycin D (Figure 6, Panel C), in contrast, are examples of intercalative agents. The classic intercalator EtBr inserts between base pairs without interfering with H-bonding, while the cationic substituent interacts with the minor groove. Binding to DNA results in the unwinding of the DNA double helix by about $26^\circ$, creating an opening of about $3.4 \text{ Å}$. This unwinding causes a structural change in the DNA strand which can lead to inhibition of transcription and DNA repair mechanisms. EtBr is widely considered a mutagen. Actinomycin D is also known to intercalate between GC rich regions of DNA with high specificity. Copper complexes are also known to be effective DNA groove binding and intercalating agents as outlined in section 1.5 below.
Figure 6 DNA Minor groove binders and intercalators: Panel A) Graphical representation of netropsin bound to the DNA minor groove. Panel B) Chemical structures of the minor groove binding agents Hoechst 33258 & netropsin. Panel C) Chemical structures of ethidium bromide and actinomycin D.

1.5 Copper

Copper, atomic number 29, is a soft and ductile metal, which forms a rich variety of compounds with a diverse range of ligands. Cu is an essential metal found in concentrations up to a few hundred parts per million (ppm) in numerous human organs and tissues.\[^{32}\] Cu in biological systems is involved in reduction-oxidation or redox type reactions where Cu-containing biological molecules react directly with molecular oxygen, producing free radicals.\[^{33}\] The chemistry of Cu is predominantly in the +1 or +2 oxidation state, i.e. cuprous and cupric states respectively. Due to the relative ease of reduction & oxidation, Cu plays an important role in the activity of several enzymes, crucial to a broad range of functions.\[^{34}\]

1.5.1 Copper proteins

Copper, when absorbed through the stomach, binds to serum albumin and is transported to the liver. Once in the liver, Cu is distributed to metabolically active sites throughout the body by mainly ceruloplasmin and other less
quantitively important Cu binders.\textsuperscript{[34-35]} To avoid cellular toxicity, free Cu is incorporated into chaperones and proteins.\textsuperscript{[35b]}

CuZn-superoxide dismutase (CuZnSOD), Table 1, for example, is a protein involved in anti-oxidation; CuZnSOD catalyses the dismutation (partitioning) of superoxide (O$_2^\cdot$\textsuperscript{−}) into molecular oxygen (O$_2$) or hydrogen peroxide (H$_2$O$_2$) (Scheme 2). In eukaryotic cells there are two intracellular and one extracellular forms of SOD. SOD1 is the major intracellular form and is present in the cytoplasm of a number of cell types examined.\textsuperscript{[36]} At the heart of the catalytic centre of CuZnSOD are Cu and Zn ions bridged together via the imidazole ring of histidine 61 (His-61). The Zn ion is coordinated by the nitrogen of three separate His residues and an oxygen of one aspartate (Asp) residue in a distorted tetrahedral geometry while Cu is coordinated via the nitrogen atoms of four His residues and a water molecule in a distorted square pyramidal geometry.\textsuperscript{[36]} The reaction in the enzyme active site is a catalytic process which involves reduction and oxidation of Cu producing O$_2$ and H$_2$O$_2$, Scheme 2.\textsuperscript{[37]}

\[
\text{Cu(II)-Zn-SOD} + \text{O}_2^\cdot \rightarrow \text{Cu(I)-Zn-SOD} + \text{O}_2
\]

\[
\text{Cu(I)-Zn-SOD} + \text{O}_2^\cdot \rightarrow \text{Cu(II)-Zn-SOD} + \text{H}_2\text{O}_2
\]

Overall reaction: \[
\text{O}_2^\cdot + \text{O}_2^\cdot + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

Scheme 2 Overview of the reaction in the catalytic site of CuZnSOD.

Other important Cu-containing proteins include dopamine-β-monoxygenase, Table 1, which is an enzyme involved in the biosynthesis of catecholamine playing a role in neurotransmission regulation.\textsuperscript{[38]} Cytochrome c oxidase, Table 1, is a Cu-containing enzyme involved in the last step of the electron transport chain where it converts O$_2$ to two molecules of water, thus playing a pivotal role in cellular respiration.\textsuperscript{[39]}
Table 1 Selected Cu-containing proteins and their functions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>Mitochondrial respiration</td>
</tr>
<tr>
<td>Cu Zn-SOD*</td>
<td>Anti-oxidant</td>
</tr>
<tr>
<td>Dopamine-β-monoxygenase</td>
<td>Catecholamine biosynthesis</td>
</tr>
</tbody>
</table>

*Cu Zn-SOD: Cu-Zn superoxide dismutase

While Cu plays an integral part in many metabolic roles in the body, elevated or reduced levels of Cu can be toxic to the body leading to diseases, mainly Wilson’s disease and Menkes syndrome.

1.5.2 Copper diseases

Wilson’s disease is an autosomal recessive disorder caused by excessive accumulation of Cu in the liver. Wilson’s disease was first described in 1912. A disruption in the function of the Cu transporter protein, ATPase, has been identified to be the major cause of this disease in patients. More than 190 mutations have been identified in the ATP7B gene which encodes for these Cu transporter proteins. Disrupting these proteins can prevent or reduce the transport of Cu out of the cell.

Menkes’ syndrome, in contrast, is a disorder caused by a deficiency of Cu uptake in the body. This disorder is caused by a mutation in the ATP7A gene which encodes for several domains for ATPase function including Cu binding. In Menkes syndrome, these mutations result in poor distribution of Cu where Cu then accumulates in tissues such as the small intestine and kidneys, whereas tissues such as the brain have unusually low levels. This decrease in Cu levels reduces the activity of a number of key Cu enzymes which are
necessary for the structure and function of bone, skin, blood vessels and the nervous system.\textsuperscript{[43]}

While Cu undoubtedly plays an integral role as an essential metal found in numerous human organs and tissues, Cu complexes in their own right have received considerable attention also as potential therapeutic agents.

1.5.3 Copper complexes as chemical nuclease agents $[\text{Cu(Phen)}_2]^{2+}$

$[\text{Cu(Phen)}_2]^{2+}$ (where Phen is 1,10-phenanthroline), herein referred to as Cu-Phen, was the first chemical nuclease agent, reported by Sigman \textit{et al.} in 1979.\textsuperscript{[44]} Cu-Phen binds B-DNA via intercalation where one of the Phen ligands intercalates in A-T rich regions in the DNA minor groove while the second Phen makes secondary non-covalent contact in the same minor groove.\textsuperscript{[45]} Metal ions such as Cu(II) & Fe(II) can react with $\text{H}_2\text{O}_2$ via Fenton chemistry to produce reactive oxygen species (ROS) resulting in oxidative DNA damage. The proposed mechanism of DNA cleavage by Cu-Phen consists of a one electron transfer reaction from Cu-Phen with formation of ROS and an unknown Cu oxo-species responsible for DNA damage (Scheme 3).\textsuperscript{[44, 46]}

\begin{equation*}
2 \text{[Cu(Phen)}_2]^{2+} + 2\text{RS}^- \rightarrow 2 \text{[Cu(Phen)}_2]^+ \hspace{1cm} \text{RS-SR}
\end{equation*}

\begin{equation*}
2 \text{[Cu(Phen)}_2]^+ + 2\text{O}_2 \rightarrow 2 \text{[Cu(Phen)}_2]^{2+} + 2\text{O}_2^- \\
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\end{equation*}

$\text{[Cu(Phen)}_2]^+ + \text{H}_2\text{O}_2 \rightarrow \text{[Cu-"oxo"(Phen)}_2]^+$

$\text{[Cu-"oxo"(Phen)}_2]^+ + \text{DNA} \rightarrow \text{[Cu(Phen)}_2]^{2+} + \text{DNA damage}$

\textit{Scheme 3} Proposed formation of reactive copper-oxo species \textit{via} Fenton Chemistry\textsuperscript{[47]}
More specifically, the Cu-Phen first binds DNA; typically to A-T rich regions of the DNA minor groove.\textsuperscript{[44]} DNA damage is initiated by abstraction of a hydrogen atom on the DNA deoxyribose ring predominantly at the C1’ position.\textsuperscript{[48]} Current evidence for the mechanism of DNA strand damage (Figure 7) by Cu-Phen is that hydrogen atom abstraction at C1’ occurs (1), followed by formation of a peroxyl radical intermediate (2), which expels superoxide (O$_2^-$)\textsuperscript{[49]} generating a 2’ deoxyribonolactone intermediate (4). In a pivotal study by Greenberg \textit{et al.}\textsuperscript{[45, 50]} Cu-Phen was found to act as a general base catalyst where direct strand damage was identified via β-elimination of the 2’ intermediate (4). In the final step, the 3’-furanone (5) is converted to the free 5-methylene furanone (6). Kellett \textit{et al.} have also found Cu-Phen to generate 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxo-dG – Figure 8) in plasmid DNA.\textsuperscript{[51]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Mechanism of oxidative DNA damage by Cu-Phen: Panel A: Chemical structure of [Cu(Phen)]$^{2+}$ (Cu-Phen) and numbering system of the deoxyribose sugar. Panel B: Proposed mechanism for DNA scission by Cu-Phen. The first step is an oxidation reaction at C1’. The second step involves an elimination reaction that yields 5-methylene-2(5H)-furanone\textsuperscript{[47]}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Generation of 8-Oxo-dG by Cu-Phen}
\end{figure}
Cu-Phen only cleaves DNA in the presence of exogenous oxidant or reductant. The Kellett group have developed a number of ‘self-activating’ Cu(II) complexes (Figure 9) which can cause DNA cleavage in the absence of endogenous reductant.\(^5\) These complexes have also been shown to possess significant *in vitro* cytotoxic activity in a panel of cancerous cell lines.\(^5\)

Figure 9 Chemical structures of ‘self-activating’ chemical nucleases developed by Kellett et al. (Ph = Phthalic acid; isoPh = isophthalic acid; Terph = Terephthalic acid; oda = octanedioic acid).

1.5.4 Cu complexes with potential intercalative properties

Cu-Phen is known to intercalate B-DNA (section 1.5.3); this complex has however only moderate DNA binding affinity with a reported apparent binding constant \(K_b\) of \(2.7 \times 10^3\) M\(^{-1}\) for calf thymus DNA (ctDNA).\(^5\) In order to improve the DNA binding affinity of Cu(II) complexes, one strategy is to replace the Phen ligands with modified \(N,N'\)-diimine ligands known to have intercalative properties as well as DNA cleavage properties under oxidative conditions. For example, \([Cu(DPQ)]^{2+}\) where DPQ is Dipyrido[3,2-\(d\):2',3'-\(f\)]quinoxaline) and \([Cu(DPPZ)]^{2+}\) where DPPZ is dipyrido[3,2-\(a\):2',3'-\(c\)]phenazine (Figure 10) have \(K_b\) values of \(4.5 \times 10^4\) M\(^{-1}\) and \(2.0 \times 10^4\) M\(^{-1}\) respectively, thus providing evidence that extension of the aromatic ring of Phen can greatly enhance the intercalative properties of the resulting complexes.
Similarly, there have been a number of studies focusing on the interaction of Cu(II) complexes containing at least one $N,N'$-diimine ligand (Figure 11). With the exception of Figure 11 complex 9, all others shown in Figure 11 were found to intercalate DNA. Complexes containing 2,2'-bipyridine (bipy, Figure 10), 3,4,7,8-tetramethyl-1,10-phenanthroline (tmp, Figure 10) phen and dpq (Figure 10) prefer intercalation within the minor groove, whereas those containing dipyrido[3,2-a:2',3'-c]phenazine (DPPZ) and 11,12-dimethyldipyrido[3,2-a:2',3'-c]phenazine (dmdppz, Figure 10) prefer intercalation within the major groove.\cite{50, 53-54}

Interestingly, complex 9 (Figure 11) showed preferential binding towards A-T versus G-C rich regions of DNA with $K_b = 1.8 \times 10^6 \text{M}^{-1}$ (poly (dA)- (d(T))) and $6.0 \times 10^5 \text{M}^{-1}$ (poly (dG)- (dC)); the $K_b$ for ctDNA was found to be $2.0 \times 10^6 \text{M}^{-1}$. 

**Figure 10 Designer DNA intercalating ligands:** Chemical structures of modified diimine $N,N'$-intercalating ligands.
Figure 11 Selected Cu complexes containing \(N,N'\)-diimine bases such as bipy, phen, dpq, dpdz and tmp with potential intercalative properties on B-DNA. Data used in the comparison in section 1.5.4:

Kellett et al. have also reported the synthesis and DNA binding properties of a series of \([\text{Cu(Phen)}(X)]^{2+}\) (where \(X = \text{DPQ, DPPZ or benzi[j]dipyridophenazine (DPPN)}\). DNA binding experiments suggested that these complexes intercalate at both the major and minor groove of ctDNA with preferential stabilization of G-C rich DNA.\[^{[56]}\] To date, the Cu(II) complexes Cu-DPQ-Phen and Cu-DPPZ-Phen have the highest known binding constants towards ctDNA (\(K_{\text{app}}\) (apparent binding constant) = 3.04 \(\times\) \(10^7\) M and 2.58 \(\times\) \(10^7\) M(bp\(^{-1}\)) Cu-DPQ-Phen and Cu-DPPZ-Phen respectively).\[^{[56]}\]
1.5.5 Cu complexes with potential minor groove interactions

Meuiner et al. reported on the rational design and DNA cleavage activity of a minor groove binder-chemical nuclease Cu(II) conjugate.\[^{57}\] This complex consists of a derivative of the minor groove binder (distamycin) conjugated to the chemical nuclease 3-Clip-Phen (Figure 13). Distamycin is a tri-N-methylpyrrole derivative that tightly binds to the DNA minor groove through a combination of electrostatic interactions, H-bonds, and van der Waals' contacts, where it preferentially binds five successive A-T base pairs.\[^{58}\] 3-Clip-Phen is a derivative of Cu-Phen in which the two Phen ligands are linked together at the 3'C of Phen via a serinol bridge. The oxidative nuclease activity of the Cu(II) complex of 3-Clip-Phen, \([\text{Cu}(3\text{-Clip-Phen})]^{2+}\), was found to be 60 fold higher than \([\text{Cu}(\text{Phen})]^{2+}\).\[^{59}\] This complex was shown to have sequence selective cleavage of DNA where oxidative DNA damage was observed at successive A-T base pairs.\[^{60}\]
1.6 Cu(II) complexes as drug agents

As discussed in section 1.3, due to the shortcomings of classical Pt drugs such as resistance and toxicity issues, there has been a surge of interest in the development of novel non-Pt-based drug candidates; the rationale being that by changing the metal, you change the chemistry and thus change the mechanism of action and, in so doing, alter the biological activity. One might therefore reasonably expect that non-Pt-based drugs might potentially overcome some of the drawbacks associated with existing therapies.

The versatility of Cu complexes, such as their biologically accessible redox states, their chemical nuclease activities, and their cationic charged complex properties\[^{26}\] has led to considerable interest in the development of Cu(II) drugs as anti-cancer agents. It has been shown also that Cu levels are amplified in a range of cancers such as breast, cervix, liver, lung, Hodgkin’s lymphoma, leukemia, GI tract cancer and brain tumours.\[^{61}\] The metal chelating ligand, Phen, either as a single-agent or incorporated into a metal complex, has been shown to exhibit excellent \textit{in vitro} anti-fungal\[^{62}\] anti-bacterial\[^{63}\] and anti-cancer properties.\[^{64}\] The therapeutic potential of Cu(II)-Phen complexes and their derivatives have been actively explored and a number of examples have been reported in the literature.

Probably the most well-known are a series of over 100 Cu(II) complexes which have been developed, registered and patented under the name of Casiopeínas® for potential use as anti-cancer therapeutics.\[^{65}\] These complexes are mixed chelate complexes with a general formula of \([\text{Cu}(A)(B)](\text{NO}_3)\) (A=\text{N,N’}-diamine ligand derivative of Phen or Bipy, B = N-O or O-O-donor such as aminoacidates or acetylacetonate). The most promising candidates to date have been \([\text{Cu}(\text{glycinate})(4,7\text{-dimethyl})\cdot 1,10\text{-phenanthroline})(\text{H}_2\text{O})](\text{NO}_3)\) (Cas-II-gly) and \([\text{Cu}(\text{acetylacetonato})(4,4’\text{-dimethyl}-2,2’\text{-bipyridine})](\text{NO}_3)\) (Cas III-ia) (Figure 14). Both have shown promising chemotherapeutic potential on a number of cancerous lines both \textit{in vitro} and \textit{in vivo}.

Despite numerous studies, the mechanism of action of these complexes is not fully understood. Cas-II-gly has been shown to interact with DNA through intercalation or adduct formation. Both have also been shown to induce mitochondrial toxicity and oxidative DNA damage after ROS generation. Encouragingly, both of these complexes have entered phase I clinical trials.

Figure 14 Chemical structures of Cu(II) complexes of the Casiopeinias series, Cas-II-Gly and Cas-III-ia.

Kellett et al. reported on a number of examples of modifications of the Cu-Phen scaffold for enhancement of DNA binding, cytotoxicity and chemical nuclease activity (Figure 15). Complexes of the type [Cu(Phen)(B)]^{2+} (B=DPQ, DPPZ or DPPN, Figure 15, panel C) have been shown to have \textit{in vitro} cytotoxic activity on a cisplatin resistant ovarian cancerous cell line (SK-OV-3); notably Cu-DPPZ-Phen was found to have on par cytotoxic activity to the clinical agent doxorubicin, in the same cell line.
Figure 15 Cu(II) complexes as drug agents: Recent examples of Cu(II) complexes as potential therapeutic agents: Panel A: Cu-Phen analogues incorporating inner sphere O,O’ coordinative ligands\(^{[71]}\), Panel B: Cu-Ph (Ph = Phthalic acid) as a lead drug agent, Panel C: Cu(II) complexes with extended π symmetry for enhanced cytotoxic and intercalative properties\(^{[56]}\).

The presence of pendant carboxylate groups in the Cu-Phen cation was found to enhance the cytotoxic activity within two human prostate cancerous cell lines (PC3 & PNT1A) (Figure 15, panel A).\(^{[71]}\) The in vitro anti-fungal and anti-bacterial properties of these complexes were also investigated across an extensive panel of pathogenic microbes, where Cu-Iso-Phen (Figure 15 panel A) was found to be highly active against gram-positive and gram-negative bacteria, as well as filamentous and non-filamentous fungi.\(^{[71]}\)

The complex Cu-Ph (Figure 15, Panel B) exhibited a broad spectrum of activity against breast (MF-7), colon (HT29), prostate (DU145) and ovarian (SK-OV-3) cancerous cell lines.\(^{[72]}\) NCI-60 anti-cancer drug screen results show that Cu-Ph exhibits low μM activity particularly against melanoma and renal sensitive cell lines.\(^{[73]}\) An array of cytotoxic and mechanistic
experiments were conducted in order to investigate the mechanism of action of Cu-Ph. The use of ROS specific scavengers both in vitro and ex vitro found superoxide was the predominant species which mediates oxidative DNA damage.\textsuperscript{[56]} Cu-Ph has also been shown to possess multi-modal targeting properties with loss of membrane potential and detection of apoptotic bodies in SK-OV-3 cells.

While DNA is clearly a molecular target receiving considerable attention, chromatin itself, into which DNA is packaged, is another related target under intense investigation.

### 1.7 Chromatin as a drug target

In cells, DNA is packaged in a macromolecule known as chromatin, the fundamental building blocks of which are nucleosomes.\textsuperscript{[74]} Two copies of each histone protein H2A, H2B, H3 and H4 are assembled into an octamer which has 145-147 DNA base pairs wrapped around it to form a nucleosome core, where each nucleosome occurs every 200 ± 40 base pairs (bp).\textsuperscript{[74-75]} The repeating nucleosome structures are assembled into highly ordered helices via the linker histone, H1.\textsuperscript{[76]} Packaging of histones in this way protects DNA from not only damage but also controls gene expression.

#### 1.7.1 Histone acetyltransferases/Histone deacetylases

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two sets of enzymes which work in harmony to control chromatin structure and thus function. HATs acetylate lysine residues of histone proteins (H2A, H2B, H3 and H4) while HDACs, as the name suggests, deacetylate these lysine residues (Figure 16). At physiological pH, in the deacetylated state, histone lysine side chain amino groups are positively charged leading to
electrostatic interactions with the negative DNA backbone. This results in a closing of the chromatin structure, preventing transcription factors from accessing DNA and thus downregulating transcription. In contrast, acetylation of these lysine amino acid residues by HATs results in the generation of neutral lysine side chains (Figure 16, Panel B). This reduces electrostatic interaction with DNA\textsuperscript{[77]} which leads to an opening of the chromatin structure, thus allowing transcription factors access DNA and causing upregulation of transcription.

**Figure 16 Histone and HDAC/HAT function:** Panel A: X-ray crystal structure of histone with protruding lysine residues\textsuperscript{[78]}. Panel B: Main mechanism of acetylation/deacetylation of HAT’s & HDAC’s.

In humans, 18 HDACs have been identified and can be sub-divided into 4 classes:

Class *I*: namely HDAC 1, 2, 3 and 8, with molecular weights of 22–55 kDa and all of which are homologous in their catalytic sites;

*Class Ila*: namely HDAC 4, 5, 7 and 9 which are larger molecules with molecular weights between 120 kDa and 135 kDa;
Class IIb: includes HDAC 6 and 10, both of which contain two catalytic sites;

Class IV: namely HDAC 11. All HDACs of the ‘classic family’ require a Zn\textsuperscript{2+} ion in their enzyme active pocket for deacetylase activity.

1.7.2 Suberoylanilide hydroxamic acid as a HDAC Inhibitor

In the late 1990s a link between HDAC inhibition and cancer cell progression was identified.\[79\] This sparked an interest in the design and development of HDAC inhibitors for clinical use where a number of structurally diverse HDAC inhibitors (HDACi) have emerged in the literature.\[80\] In fact, more than 350 clinical trials involving HDAC inhibitors have been undertaken or are being carried out against various diseases, both as monotherapy and in combination with another agent.\[81\]

Suberoylanilide hydroxamic acid (SAHA, U.S. trade name Zolinza\textsuperscript{®}) was patented in 1993.\[82\] When assessed using the NCI-60 anti-cancer drug screen, SAHA was found to have broad spectrum \textit{in vitro} cytotoxic activity across a range of cancerous cell lines with an overall mean Growth Inhibition 50\% (GI\textsubscript{50}) value of 0.94 \textmu M.\[83\] SAHA has also been found to possess broad spectrum \textit{in vivo} activity in a range of solid, malignant and haematological tumours, examples including: B-cell lymphoma,\[84\] prostate,\[85\] thyroid,\[86\] glioma\[87\] and ovarian\[88\] cancers. SAHA has also recently been shown to possess selective cytotoxicity towards mutant p53 cancerous cells.\[89\]

SAHA was the first hydroxamate-based HDACi to receive US FDA approval - in late 2006 it entered the clinic as a treatment for patients with progressive, persistent or recurrent cutaneous T-cell lymphoma (CTCL) following two systemic therapies.\[90\] The chemical structure of SAHA (Figure 17 Panel B) consists of a benzamide protein recognition domain, a C7 alkyl linker and a Zn(II) hydroxamic acid binding domain. SAHA is considered a pan HDAC inhibitor; demonstrating HDAC inhibitory activity against a range of HDAC
classes (class I, II & IV). X-ray crystallographic analysis of SAHA bound to various human HDAC proteins has shown SAHA bound to the Zn(II) ion at the catalytic site via its hydroxamate moiety while the C7-linker fits into a narrow channel and the phenyl group serving to dock the inhibitor by acting as a protein surface recognition domain. Access by its natural substrate, lysine, into its active site, is therefore denied.

**Figure 17 HDACi properties of SAHA:** Panel A) X-ray crystal structure of SAHA bound to the HDAC active site; Panel B) Chemical structure of SAHA.

In phase I and phase II clinical trials, SAHA administered alone or in combination therapy was found to be reasonably tolerated by patients with myelosuppression, GI toxicity and fatigue being cited as dose-limiting side effects.\(^{[92]}\)

Our group has been interested for some time in the rational design, synthesis and biological evaluation of multi-functional drug conjugates incorporating, for example, DNA-binding metal ions such as Pt and enzyme inhibitors such as those of HDACs. Recently, we reported two such drug candidates; cis-[Pt(NH\(_3\))\(_2\)(malSAHA-\text{2H})] and cis-[Pt(NH\(_3\))\(_2\)(mal-p-Bel-\text{2H})] (where Bel is Belinostat, another clinically used hydroxamate-based HDACi).\(^{[93]}\) These complexes (Figure 18) consist of a synthetically modified HDACi (SAHA or Belinostat respectively) tethered to a Pt(II) metal centre via an O,O'-malonato
Both have been shown to possess potent in vitro cytotoxicity - on par with cisplatin. \textit{cis}-[Pt(NH$_3$)$_2$(malSAHA$_{2H}$)] has also been found to be highly cytotoxic in an in vivo ovarian xenograft tumour model. HDAC inhibitors such as SAHA and Belinostat are also known to be selective for tumour cells. The presence of the conjugated HDACi, malSAHA, in \textit{cis}-[Pt(NH$_3$)$_2$(malSAHA$_{2H}$)], appears to significantly enhance Pt accumulation within cancerous cells.$^{[94]}$ \textit{cis}-[Pt(NH$_3$)$_2$(malSAHA$_{2H}$)] was also

found to be significantly less toxic in two normal cell lines thus supporting the hypothesis that the presence of the HDAC inhibitor, with its known selectivity for tumour cells, may well confer selectivity to metal-HDAC inhibitor conjugates.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{multi-functional_pt_ii_drug_conjugates.png}
\caption{Multi-functional Pt(II) drug conjugates: Synthetic rationale and chemical structures of multi-functional Pt(II) drug conjugates. Data taken from previously reported results$^{[22, 93]}$}
\end{figure}
1.8 Outline & Scope of this Thesis

There have been no reports to date of Cu(II) complexes in which the Cu(II) ion is coordinated to both (i) a HDAC inhibitor and (ii) various designer DNA intercalating ligands. We sought to fill this void by generating innovative multi-functional Cu(II) complexes possessing both HDAC inhibitory and DNA cleavage properties. The aim of this PhD thesis is to describe the synthesis and in vitro pharmacological properties of a novel series of such Cu(II) conjugates (Figure 19).

We hypothesized that these complexes may be selectively uptaken by cancerous cells due to (i) the presence of the HDAC inhibitor, with known selectivity for cancerous cells over normal cells[89] and (ii) the requirement of endogenous metals such as Cu(II) for cancerous cells (section 1.6). Upon entry into the more reducing environment of cancerous cells, we hypothesised that these multi-functional drug candidates may be activated by reduction from Cu(II) to Cu(I) with concomitant release of both the HDAC inhibitor and the Cu-N,N'-DNA intercalating moieties – thus free to inhibit HDAC enzymes and execute oxidative DNA damage respectively.

Figure 19 Rational-based CuII drug design: Chemical structures of target complexes.

Herein we describe the synthesis and characterisation of a library of Cu-SAHA-Phen-type complexes. Their HDAC inhibitory activity, cytotoxicity, DNA binding and chemical nuclease properties were investigated with a view
to generating a structure-activity relationship and are also herein described. Given that Cu(II) complexes have also been shown to possess anti-bacterial and anti-fungal activity, we present herein preliminary results regarding the anti-bacterial and anti-fungal properties of these novel Cu(II) complexes.

Chapter 1 – Chapter 1 provides a brief introduction of the work including previous results in the literature and rationale for the design of the Cu(II) chemotypes described.

Chapter 2 – Chapter 2 describes the synthesis and characterisation of the Cu(II)-SAHA-Phen chemotypes. We also attempted to investigate the behaviour of these complexes in solution, the results of which are also described.

Chapter 3 – Chapter 3 describes the in vitro cytotoxic activity of the Cu(II)-SAHA-Phen chemotypes across an extensive panel of human cancerous, fungal and microbial cell lines. We also describe their in vitro HDAC inhibitory activity in the human ovarian cancer cell line SK-OV-3.

Chapter 4 – Chapter 4 outlines the DNA binding properties of the Cu(II)-SAHA-Phen chemotypes and preliminary investigations into their intercalative modes. We also describe their DNA cleavage properties and highlight, following ROS scavenging studies, potential ROS species involved in DNA scission.

Chapter 5 – In Chapter 5 we discuss the body of work as a whole with suggestions for future studies.
Our ultimate aim was to generate innovative anti-cancer metallodrug candidates for further development.
1.9 Chapter 1 References


Chapter 2 - Synthesis and Characterisation of a Library of Novel Multi-Functional Copper(II) Complexes Incorporating Histone Deacetylase Inhibitor and DNA Intercalating Moieties
2.1 Introduction

As described in chapter 1, coordination of Cu(II) ions to phenanthrene-based intercalating ligands has produced an array of potential drug candidates with promising DNA binding, chemical nuclease activity and cytotoxicity against a range of tumour cell lines. An alternative strategy to enhance the activity and safety profile of metallodrugs is to conjugate metal ions to clinically used drug molecules or molecules which, in their own right, have demonstrated potent activity. Cu(II) complexes incorporating non-steroidal anti-inflammatory drugs (NSAIDs), for instance, have been reported.[1]

Our group has been interested for some time in the rational design, synthesis and biological evaluation of multi-functional drug conjugates incorporating, for example, DNA-binding metal ions such as Pt and enzyme inhibitors such as those of HDACs with two lead drug candidates currently undergoing further development, namely \( \text{cis-[Pt(NH}_3)_2\text{(malSAHA} \cdot 2\text{H})]^{[2]} \) and \( \text{cis-[Pt(NH}_3)_2\text{(mal-p-Bel} \cdot 2\text{H})]^{[2b]} \). HDAC inhibitors represent an attractive target in that they have been shown to have potent \textit{in vitro} and \textit{in vivo} cytotoxicity against a range of tumours with two hydroxamate-based HDACis, namely SAHA and Belinostat, already in clinical use as anti-cancer agents.

There have been no reports to date of Cu(II) complexes in which the Cu(II) ion is coordinated to both (i) a HDAC inhibitor and (ii) various designer DNA intercalating ligands. We sought to fill this void by generating innovative multi-functional Cu(II) complexes possessing both HDAC inhibitory and DNA cleavage properties. We hypothesised that, upon entry into the more reducing environment of cancerous cells, these multi-functional drug candidates may be activated by reduction from Cu(II) to Cu(I) with concomitant release of both the HDAC inhibitor and the Cu-\( N,N' \)-DNA intercalating moieties – thus free to inhibit HDAC enzymes and execute oxidative DNA damage respectively.
Herein, we describe the synthesis, characterisation and speciation studies of the first examples of such multi-functional Cu(II) complexes (Figure 20) incorporating SAHA as a HDACi and Phen, Phendio, Bipy, DPQ and DPPZ as DNA intercalating moieties. Speciation studies were conducted in order to provide insight into the stability and behaviour of these systems in solution and to provide preliminary evidence of HDAC inhibitor release at low pH.

![Figure 20 Rational-based Cu(II) drug design: Chemical structures of target complexes.](image)

**2.2 Materials and Methods**

**2.2.1 General Chemicals**

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Arklow, Ireland) as reagent grade and used without further purification. SAHA,[3] 1,10-Phenanthroline-5,6-dione (Phendione),[4] Dipyrido[3,2-f:2',3'-h]quinoxaline (DPQ)[4] and Dipyrido[3,2-a:2',3'-c]phenazine (DPPZ)[4] were prepared by previously reported methods.

**2.2.2 Characterisation**

$^1$H NMR spectra were recorded on a Bruker 400MHz instrument and spectra were analysed using MestReNova® software. UV-Vis spectroscopy was performed on an Agilent Cary 100 dual beam spectrophotometer equipped with a 6 x 6 peltier multicell system. Samples for molar extinction coefficient measurements were prepared at 1 mM in DMSO, molar extinction coefficients were calculated using the following equation: $\varepsilon = \text{Abs @ } \lambda_{\text{max}} \div (0.001 \text{ M}) \times (1 \text{ cm})$. Infra-red spectra were recorded on a PerkinElmer Spectrum 100 spectrometer equipped with a universal ATR accessory and the spectra analysed using SPECTRUM software. Electrospray ionisation mass spectrometry (ESI-MS) experiments were carried out on an Advion
Expression Compact Mass Spectrometer, ESI-MS samples were prepared (<0.1 mg/ml) in methanol (MeOH) as solvent. Elemental analysis (C, H, N) were carried out on an Exeter Analytical CE440 elemental analyser. Cu analysis was carried out on a Varian 55B atomic absorption spectrometer. Cl analysis was determined via combustion in an oxygen flask followed by titration with mercuric nitrate. Elemental analysis experiments were conducted in the School of Chemistry and Chemical Biology, University College Dublin.

2.2.3 Ligand syntheses

Suberanilic acid was prepared by a procedure reported by Gore et al[3]. A mixture of 1,1'-carbonyldiimidazole (CDI) (11.68g, 0.5eq, 72.06mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (23.79g, 0.8eq, 115.296mmol) in the minimum amount of tetrahydrofuran (THF) was stirred for 1 hour at 25-30°C. Suberic acid (25g, 1eq, 144.12mmol) and aniline (13.2ml, 1eq, 144.12mmol) in the minimum volume of THF was added and the mixture stirred for a further 16-20 hours. The solid by-product was removed by filtration and the filtrate was concentrated in vacuo at 50°C. The solid residue obtained was treated with a solution of KOH (2eq) in water and stirred for 30 minutes at 25-30°C and any solid by-product formed was removed by filtration. The filtrate obtained was heated at 60°C for 3-4 hours and cooled to 20°C before addition of an aqueous solution of HCl (17.5%). The mixture was stirred for 30 minutes and the solid filtered, washed with water (2x20ml) and dried under vacuum. Yield 18.75g, 52%. ^1H NMR (400MHz, DMSO d6) δ 11.92 (1H, s, OH) 9.77 (1H, s, NH) 7.51 (2H, d, Ar2,6-H) 7.21 (2H, t, Ar3,5-H) 6.94 (1H, t, Ar4-H), 2.23 (2H, m, C2,7-H), 2.13 (2H, m, C2,7-H), 1.45 (4H, m, C3,6-H), 1.24 (4H, m, C4,5-H).
Scheme 5 Synthesis of SAHA

SAHA was prepared by a procedure reported by Gore et al.\textsuperscript{[3]} Suberanilic acid (3.3 g, 1 eq, 13.24 mmol) was dissolved in 7 ml of \(N,N\)-Dimethylformamide (DMF). CDI (4.32 g, 2 eq, 26.48 mmol) was added and stirred at 25 - 30 °C for 30 minutes. Hydroxylamine hydrochloride (3.71 g, 4 eq, 52.96 mmol) was added and the solution was for 30 minutes at 25-30°C. De-ionised water (30 ml) was then added and the mixture stirred for 2 hours at 25 – 30 °C. The precipitate was filtered, washed with water (2 x 30 ml) and dried under vacuum. Yield 2.18 g, 62.2%. \(^1\)H NMR (400 MHz, DMSO d\textsubscript{6}) \(\delta\) 10.31 (1H, s, NH) 9.82 (1H, s, NH) 8.63 (1H, s, OH) 7.57 (2H, d, \(\text{Ar}_{2,6}\)H) 7.27 (2H, t, \(\text{Ar}_{3,5}\)H), 7.01 (1H, t, \(\text{Ar}_{4}\)-H) 2.28 (2H, m, \(\text{C}_{2,7}\)-H), 1.94 (2H, m, \(\text{C}_{2,7}\)-H), 1.57 (2H, m, \(\text{C}_{3,6}\)-H), 1.49 (2H, m, \(\text{C}_{3,6}\)-H), 1.27 (4H, m, \(\text{C}_{4,5}\)-H).

Scheme 6 Synthesis of 1,10-Phenanthroline-5,6-dione (Phendione)

Phendione was prepared by a procedure reported by Kellett et al.\textsuperscript{[4]} 1,10-Phenanthroline (4.00 g, 22.19 mmol, 1 eq) and potassium bromide (4.00 g, 33.6 mmol, 1.5 eq) were thoroughly mixed and slowly added to an ice-cold mixture of \(\text{H}_2\text{SO}_4\) (40 mL) and \(\text{HNO}_3\) (20 mL). The solution was refluxed for 3 h at 100 °C, then cooled to room temperature, poured onto crushed ice (~400 mL), and neutralized with an aqueous NaOH solution (80.0 g per 400 mL) to a pH between 4 and 5, yielding a yellow solution. The solution was extracted with \(\text{CHCl}_3\) (in 8 x 100 mL portions). The organic phase was combined and
dried with anhydrous magnesium sulfate and then filtered before being evaporated to dryness, whereupon a bright yellow solid (4.03 g) was obtained. The crude product was recrystallized from HPLC-grade methanol. Yield: 4.03 g (86%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): 9.10 (dd, J = 4.6, 1.8 Hz, 2H, C\(_{3,8}\)-H), 8.51 (dd, J = 7.9, 1.8 Hz, 2H, C\(_{2,9}\)-H), 7.58 (dd, J = 7.9, 4.6 Hz, 2H, C\(_{1,10}\)-H).

Scheme 7 Synthesis of Dipyrido[3,2-f:2',3'-h]quinoxaline (DPQ)

DPQ was prepared by a procedure reported by Kellett \textit{et al.}\(^4\) To a solution of phendione (0.510 g, 1 eq, 2.44 mmol) in water (35 mL) was added ethylenediamine (0.70 mL, 4.3 eq, 10.47 mmol), and the resultant suspension was refluxed for 12 h at 60 °C. The resulting product was washed with water (10 mL) and minimum volume of diethyl ether. Yield: 0.372 g (66%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): 9.48 (dd, J = 8.2, 1.8 Hz, 2H, C\(_{1,15}\)-H), 9.27 (dd, J = 4.3, 1.8 Hz, 2H, C\(_{2,14}\)-H), 8.98 (s, 2H, C\(_{7,8}\)-H), 7.79 (dd, J = 8.2, 4.3 Hz, 2H, C\(_{3,15}\)-H).

Scheme 8 Synthesis of Dipyrido[3,2-a:2',3'-c]phenazine (DPPZ)

DPPZ was prepared by a procedure reported by Kellett \textit{et al.}\(^4\) A methanolic solution (20 mL) of 1,2-phenylenediamine dihydrochloride (0.640 g, 3.53 mmol) was refluxed until it was dissolved. A warm ethanolic solution of phendione (0.500 g, 2.38 mmol) was prepared (20 mL), added over the
methanolic solution, and refluxed with constant stirring for 3 h. The resulting precipitate was vacuum-filtered and recrystallized from EtOH, producing metallic-like orange filaments. Yield: 0.521 g (78%). $^1$H NMR (400 MHz, CDCl$_3$): 9.65 (dd, $J = 8.1$, 1.7 Hz, 2H, C$_{9,10}$-H), 9.26 (dd, $J = 4.5$, 1.7 Hz, 2H, C$_{8,11}$-H), 8.36 (dd, $J = 6.5$, 3.4 Hz, 2H, C$_{3,16}$-H), 7.92 (dd, $J = 6.5$, 3.4 Hz, 2H, C$_{2,17}$-H), 7.79 (dd, $J = 8.1$, 4.5 Hz, 2H, C$_{1,18}$-H).

2.2.4 Complex syntheses

Caution! Although no incidents were encountered during this work, transition metal perchlorate salts are potentially explosive and should be handled with care.

Scheme 9 Synthesis of [Cu(SAHA$_{10}$)(DPPZ)]ClO$_4$

Cu(ClO$_4$)$_2$·6H$_2$O (106.4 mg, 0.287 mmol, 1 equivalent (eq) – Limiting Reagent (LR)) was dissolved in a minimum volume of DI H$_2$O (1 ml). A hot solution of SAHA (75.9 mg, 0.287 mmol, 1 eq) in a minimum volume of MeOH (1 ml) was added to the Cu solution followed by a hot solution of DPPZ (81.04 mg, 0.287 mmol, 1 eq) in a minimum volume of MeOH (3 ml). The resulting suspension was stirred at RT for 30 min. The solid product was filtered, washed with cold DI H$_2$O, cold MeOH and dried under vacuum, Scheme 9.
Cu(ClO$_4$)$_2$·6H$_2$O (108.6 mg, 0.293 mmol, 1 eq - LR) was dissolved in a minimum volume of DI H$_2$O (1 ml). A hot solution of SAHA (77.7 mg, 0.293 mmol, 1 eq) in a minimum volume of MeOH (1 ml) was added to the copper solution followed by a hot solution of 1,10-phenanthroline-5,6-dione (Phendio) (61.7 mg, 0.293 mmol, 1 eq) in a minimum volume of hot ethanol (EtOH) (4 ml). A solution of KOH (16.4 mg, 0.293 mmol, 1 eq) in a minimum volume of DI H$_2$O (1 ml) was immediately added and the solution left to stir at room temperature for 30 minutes. The mother liquor was left to stand at RT for ~5 days. A green solid by-product was filtered and the filtrate was concentrated in vacuo. The waxy solid was suspended in DI H$_2$O, agitated and a brown solid was filtered, washed with cold DI H$_2$O, cold MeOH and dried under vacuum, Scheme 10.

Copper(II) perchlorate hexahydrate (Cu(ClO$_4$)$_2$·6H$_2$O) (100 mg, 0.270 mmol, 1eq – LR) was dissolved in a minimum volume of DI H$_2$O (1 ml). A hot solution of SAHA (71.4 mg, 0.270 mmol, 1eq) in a minimum volume of MeOH (1 ml) was added to the Cu solution followed by a 0.270 mmol (1eq) solution
of either Bipy (42.5 mg), Phen (48.7 mg) or DPQ (46.7 mg) in a minimum volume of MeOH (1 ml). A solution of KOH (15.1 mg, 0.270 mmol, 1 eq) in a minimum volume of DI H₂O (1 ml) was immediately added and the resulting suspension stirred at RT for 30 min. The solid product was filtered, washed with cold DI H₂O, cold MeOH and dried under vacuum.

[Cu(SAHA-1H)(Bipy)]ClO₄·0.5 H₂O (Cu-SAHA-Bipy): Green solid, Yield 107.3 mg, 67.7 %. C₂₄H₂₈ClCuN₄O₈ requires C - 48.08 %; H - 4.71 %; N - 9.35 %; Cu - 10.60 %; Cl - 5.91 %; found C - 48.39 %; H - 4.38 %; N - 9.30 %; Cu - 10.67 %; Cl - 5.73 %. ESI-MS ([M-H]+) (MeOH) mass-to-charge ratio (m/z): 483 ([Cu(SAHA-1H)(BIPY)]⁺) 265.2 (SAHA) 219.8 ([Cu(BIPY)]⁺). IR (ATR) cm⁻¹: 3567.39 (s,s), 2931.19, 1660.60 (s,s) 1595.55. (s,s) 1071.57 (s,br).

[Cu(SAHA-1H)(Phen)]ClO₄·0.5 H₂O (Cu-SAHA-Phen): Blue solid, Yield 90 mg, 80 %. C₂₆H₂₈ClCuN₄O₉ requires C - 48.83 %; H - 4.41 %; N - 8.76 %; Cu - 9.94 %; Cl - 5.54 %; found C - 49.25 %; H - 4.56 %; N - 8.62 %; Cu - 10.36 %; Cl - 5.44 %. IR (ATR) cm⁻¹: 3495.44, 3065.47, 1650.33 1535.02 1070.18. ESI-MS ([M-H]+) (MeOH) m/z: 506.1 ([Cu(SAHA-1H)(Phen)]⁺) 265.1 (SAHA) 243.9 ([Cu(Phen)]⁺).

[Cu(SAHA-1H)(Phendio)]ClO₄·0.5 H₂O (Cu-SAHA-Phendio): Brown solid, Yield 105 mg, 67%. C₂₆H₂₆ClCuN₄O₁₀ requires C - 47.79 %; H - 4.01 %; N - 8.57 %; Cu - 9.72 %; Cl - 5.43 %; found C - 47.55 %; H - 3.99 %; N - 8.37 %; Cu - 9.51 %; Cl - 5.21 %. IR (ATR) cm⁻¹: 3377.67, 3222.67, 1693.41, 1598.73, 1070.18.

[Cu(SAHA-1H)(DPQ)]ClO₄ (Cu-SAHA-DPQ): Grey solid, Yield 96.8 mg, 53.01 %. C₂₈H₂₇ClCuN₆O₇ requires C – 51.07 %; H - 4.13 %; N - 12.76 %; Cu - 9.65 %; Cl - 5.38 %; found C - 50.65 %; H - 3.81 %; N - 12.57 %; Cu - 9.63
%; Cl - 4.97 %. ESI-MS ([M-H]+) (MeOH) m/z: 558.1 ([Cu(SAHA-H)(DPQ)]+) 294.8 ([Cu(DPQ)]+) 265.1 (SAHA). IR (ATR) cm⁻¹: 3350.21, 3050.31, 1658.57, 1595.07, 1087.07.

[Cu(SAHA-H)(DPPZ)]ClO₄ (Cu-SAHA-DPPZ): Green solid, Yield 127 mg, 60.5%. C₃₂H₃₁ClCuN₆O₇ requires C - 54.09 %; H - 4.40 %; N - 11.83 %; Cu - 8.94 %; Cl - 4.99 % ; found C - 53.70 %; H - 3.83 %; N - 11.65 %; Cu - 9.05 %; Cl 4.80 %. ESI-MS ([M-H]+) (MeOH) m/z: 609 ([Cu(SAHA-H)(DPPZ)]+) 265.2 (SAHA). IR (KBr) cm⁻¹: 3346.37, 3071.09, 1652.96, 1592.59, 1073.17.

2.2.5 Potentiometric Titrations
Potentiometric titrations were carried out in a 50:50 w/w % (DMSO:H₂O) mixture at an ionic strength of 0.2 M (KCl) and at a constant temperature of 25 ± 0.1 °C. Carbonate free KOH solution (ca. 0.2 M), acid (HCl) and salt (KCl) in the same mixture were used as the titrant. Stock solutions of HCl were prepared from concentrated HCl and their concentrations determined by the Gran method.[5] Potentiometric measurements were carried out using a DL53 titrator (Mettler Toledo, Switzerland) equipped with a DG116 capillary glass electrode (Mettler Toledo, Switzerland) and DV1010 burette (Mettler Toledo, Switzerland). Prior to measurements, the electrode was equilibrated in the solvent mixture at room temperature for 3 days. All titrations were terminated once precipitation occurred; the collected experimental data was fitted using SUPERQUAD program.

2.3 Results & Discussion

2.3.1 Ligand syntheses
SAHA was synthesized by a protocol reported by Gore et al (Scheme 12).[3] The synthesis of SAHA involved a two-step coupling reaction; the first step involved coupling suberic acid to aniline then a second coupling of the suberanic acid precursor to hydroxylamine to from SAHA (Scheme 4) The intermediates were synthesized in excellent yield and purity. They were
characterised using elemental analysis, $^1$H and $^{13}$C NMR and IR spectroscopy as well as mass spectrometry.

![Scheme 12 Synthesis of SAHA reported by Gore et al.](image)

The ligands Phendio, DPQ and DPPZ were synthesized by a procedure reported by Kellett *et al.*[^4] (Scheme 13). Oxidation of 1,10-phenanthroline (Phen) in acidic conditions formed the Phendio intermediate. Schiff-base condensation of Phendio with etylenediamine or 1,2-phenylenediamine afforded the phenazine ligands DPPZ and DPQ respectively in high yield. $^1$H NMR and $^{13}$C NMR and IR spectroscopic characterisation was in agreement with previous literature results.[^4]

![Scheme 13 Synthesis of the quinoxaline ligand Phendio; phenazine ligands DPQ and DPPZ reported by Kellett *et al.*](image)
2.3.2 Complex Synthesis

All complexes were obtained in excellent purity and good yield. Elemental analytical results were in consistent agreement (± 0.4%) with theoretical values for all the Cu(II) complexes synthesised, Table 2. We attempted to grow crystals suitable for X-ray analysis by various methodologies (slow evaporation, recrystallisation, vapour diffusion, etc) unfortunately, we could not grow crystals suitable for X-ray analysis.

<table>
<thead>
<tr>
<th>Complex</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>Cu</th>
<th>Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-SAHA-Phen</td>
<td>49.25</td>
<td>4.56</td>
<td>8.62</td>
<td>10.36</td>
<td>5.44</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>47.55</td>
<td>3.99</td>
<td>8.37</td>
<td>9.51</td>
<td>5.21</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>48.39</td>
<td>4.38</td>
<td>9.30</td>
<td>10.67</td>
<td>5.73</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ</td>
<td>50.65</td>
<td>3.81</td>
<td>12.57</td>
<td>9.63</td>
<td>4.97</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>53.70</td>
<td>3.83</td>
<td>11.65</td>
<td>9.05</td>
<td>4.80</td>
</tr>
</tbody>
</table>

*Data represented as: measured value (theoretical value).

The parent complex [Cu(SAHA,1H)(Phen)]ClO₄ was successfully synthesised via a one pot in situ reaction. First, a solution of Cu(ClO₄)₂·6H₂O was prepared, to which a 1 molar equivalent solution of SAHA was added followed immediately by a 1 molar equivalent solution of Phen. After addition of reagent solutions, a number of immediate colour changes were observed: blue to green with addition of SAHA to Cu(ClO₄)₂·6H₂O, then green to dark blue after addition of Phen. Colour changes form blue to green and vice versa are indicative of a change in coordination environment around the Cu(II) metal centre. Addition of the Phen solution, followed by addition of a 1 molar equivalent solution of KOH to the mixture, resulted in the immediate precipitation of a blue solid.
As mentioned earlier, our aim was to synthesize a library of Cu(II) conjugates incorporating the redox active Cu(II) ion coordinated to the hydroxamic acid moiety of SAHA and various designer DNA intercalating ligands (Phen, Phendio, Bipy, DPQ and DPPZ) – Scheme 14.

**Scheme 14 DNA targeting derivitisation:** Synthetic schemes for the synthesis of Cu(II) complexes with various designer DNA intercalating ligands

The synthesis of the [Cu(SAHA$_{1.14}$)(Phen)]$^{n+}$ was challenging which required optimisation of the synthetic protocol. Our initial attempts to optimise the reaction conditions centred on first the synthesis of Cu precursors - [Cu(SAHA)]$^{n+}$ & [Cu(Phen)]$^{n+}$ then coordination of respective ligands to these precursors. The precursors which formed, i.e. [Cu(SAHA)]$^{n+}$ and [Cu(Phen)]$^{n+}$, were insoluble in numerous solvents such as MeOH, ethanol (EtOH), H$_2$O, ethyl acetate (EtOAc), hexane and acetonitrile (CH$_3$CN). Attempts were made to improve the solubility of the precursors by changing the counterion using various Cu(II) salts including Cu(NO$_3$)$_2$, Cu(SO$_4$)$_2$, Cu(OH)$_2$, CuCl$_2$ but to no avail.
All complexes were synthesized (section 2.2.4) using a similar or slightly modified procedure to that of the [Cu(SAHA-1H)(Phen)]ClO$_4$ complex. Similar colour changes during the reaction were observed, however for Cu-SAHA-DPPZ and Cu-SAHA-Phendio. The reaction protocol was slightly modified due to solubility or precipitation of the complexes. For the Cu-SAHA-DPPZ complex, upon mixing of solutions of DPPZ to the Cu(ClO$_4$)$_2$·(H$_2$O)$_6$/SAHA solution, an immediate precipitation of a green solid was observed. For the Cu-SAHA-Phendio no precipitation after addition of KOH was observed. The solution had to be left to slowly evaporate for ~ 5 days, after which the Cu-SAHA-Phendio complex was isolated by rotary evaporation from the mother liquor.

2.3.4 Chemical Characterisation - Mass Spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) is a technique in which a compound of interest is subjected to ESI which produces both parent and fragmentation ions. These ions are passed through a detector and measured as mass to charge ratio (m/z) units which, in essence, measure the molecular mass of the various ions produced. For example, in the mass spectrum of Cu-SAHA-Phen, a parent ion peak at 506.10 mass to charge ratio (m/z) was observed corresponding to [${}^{63}\text{Cu(SAHA-1H)(Phen)}^+$]. Peaks were identified at 265.1 and 243.9 m/z which equate to the SAHA-H$^+$ and [Cu(Phen)]$^+$ fragmentation products respectively. Similar results in the mass spectra of Cu-SAHA-DPQ, Cu-SAHA-DPPZ and Cu-SAHA-Bipy were found and are summarised in Table 3.
Table 3: Summarised mass spectrum peaks (ESI-MS, MeOH +ve mode)

<table>
<thead>
<tr>
<th>Complex (molecular weight)</th>
<th>Parent Ion m/z (chemical structure)</th>
<th>Molecular fragment #1 m/z (chemical structure)</th>
<th>Molecular fragment #2 m/z (chemical structure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-SAHA-Phen (606.52)</td>
<td>506.10 ([Cu(SAHA$_{1H}$)(Phen)]$^+$)</td>
<td>265.1 (SAHA + H)</td>
<td>243.9 ([Cu(Phen)]$^+$)</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy (582.50)</td>
<td>483.00 ([Cu(SAHA$_{1H}$)(Bipy)]$^+$)</td>
<td>265.1 (SAHA + H)</td>
<td>219.8 ([Cu(Bipy)]$^+$)</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ (658.56)</td>
<td>558.10 ([Cu(SAHA$_{1H}$)(DPQ)]$^+$)</td>
<td>294.8 ([Cu(DPQ)]$^+$)</td>
<td>265.1 (SAHA + H)</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ (708.62)</td>
<td>609.00 ([Cu(SAHA$_{1H}$)(DPPZ)]$^+$)</td>
<td>265.2 (SAHA + H)</td>
<td>Not observed</td>
</tr>
</tbody>
</table>

Several attempts to obtain a mass spectrum of the Cu-SAHA-Phendio complex were made but no peaks corresponding to the parent ion or corresponding fragments were identified. This may possibly be due complex degradation during analysis.

2.3.5 Chemical Characterisation - IR

IR spectroscopy is most routinely employed to study vibrational modes of bonds in molecules.[1] IR is also a useful technique for assisting in the assignment of coordination modes of metal-hydroxamato complexes. Upon complexation to a metal centre, changes in vibration frequency result in a shift of IR absorption bands, indicative of coordination of a ligand to a metal ion. In the IR spectrum of Cu-SAHA-Phen, for example, there is a clear shift of the $\nu$(C=O) from 1630.25 cm$^{-1}$ (free ligand) to 1598.73 cm$^{-1}$ (Cu(II) complex). This lowering of wavenumber by $\sim$40 cm$^{-1}$ is indicative of O,O$'$ hydroxamato coordination.[6] Characteristic bands for $\nu$(N-O) also shifted from lower to higher wavenumber, i.e. 960.23 cm$^{-1}$ (free ligand) to 988.82 cm$^{-1}$ (Cu(II) complex); both observations are consistent with typical IR shifts for chelated hydroxamate groups.[7] An intense band at 1070.18 cm$^{-1}$ corresponding to the $\nu$(Cl-O) stretch of the perchlorate counter ion was also observed. Similar trends were observed in the IR spectra of all complexes synthesized, the details of which are summarised in Table 4.
Table 4: Characteristic IR stretching frequencies free ligands and complexes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\nu_{C=O}$ Hydroxamate</th>
<th>$\nu_{N-O}$ Hydroxamate</th>
<th>$\nu_{Cl-O}$ Counterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-SAHA-Phen</td>
<td>1598.73 (1630.25)</td>
<td>988.82 (899.82)</td>
<td>1070.18</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>1667.61 (1630.25)</td>
<td>997.49 (899.82)</td>
<td>1070.61</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>1595.55 (1630.25)</td>
<td>971.03 (899.82)</td>
<td>1071.57</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ</td>
<td>1596.07 (1630.25)</td>
<td>974.60 (899.82)</td>
<td>1087.87</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>1592.59 (1630.25)</td>
<td>981.26 (899.82)</td>
<td>1073.17</td>
</tr>
</tbody>
</table>

*Data represented as measured value – complex (measured value – free ligand)

2.3.6 Speciation studies

As described in sections 2.2.4 & 2.3 a series of novel multi-functional Cu(II) conjugates as potential anti-cancer agents were synthesized.

In an attempt to generate concentration distribution curves across a pH range for each of the systems synthesised, stability constants for each of the systems (i.e. Cu + N,N' ligand; Cu + SAHA; Cu + SAHA+ N,N' ligand) had first to be determined. pH potentiometric titrations were employed to measure these. During the titrations, volume increments of base are added to a titration vessel containing a metal ion and protonated ligand (HL, Scheme 15) the base deprotonates the ligand resulting in release of one or more H$^+$ in solution. This deprotonation facilitates the coordination of metal ions such as Cu(II) to the deprotonated ligand.

The pH data collected during the potentiometric titrations can be curve fitted and statistical analysis carried out to determine the stability constants for a given system. Stability constants are essentially equilibrium constants, that reflect the strength of interaction between a metal ion (M) and a ligand (L) in
the ML complex; the higher the stability constant the stronger the interaction between the two entities.

\[ M + HL \rightleftharpoons ML \]

Scheme 15 Generalised equilibrium between a metal ion (M) and protonated ligand (HL)

2.3.7 Binary pH potentiometric titrations

Due to the limited aqueous solubility of the ligands and/or Cu(II) complexes, a series of solubility tests were conducted to find the appropriate solvent mixture for the titration experiments. Titrations were ultimately carried out in 50:50 w/w (DMSO:H₂O) solvent mixture. In order to calculate relative concentration(s) of species in solution, stability constants of all systems were required including binary: \( N,N'\)-ligand + Cu; SAHA + Cu; and ternary: Cu+SAHA+\( N,N'\)-ligand. Following conditioning of the electrode, each system was calibrated by calculating the water ionisation constant (\( pK_w \)) of the solvent mixture. The calculated \( pK_w \) was 15.45 ± 0.1 which is in excellent agreement with previous reports in an identical solvent mixture.⁶

Samples for potentiometric titrations were prepared at acidic pH (approximately pH 3), with a constant ionic strength of 0.2 M KCl and at typically 1:1, 1:2 & 1:4 (Cu:\( N,N'\)-ligand/SAHA) ratios. Potentiometric titrations of stock solutions of \( N,N'\)-ligands and SAHA enabled assessment of purity and exact concentrations. Estimated protonation constants are summarized in Table 5 – for comparison purposes, literature values obtained in aqueous solution are also shown.
Table 5: Estimated protonation constants of the ligands in DMSO-water 50:50 w/w % (DMSO:H₂O) mixture and in water, I = 0.20 M (KCl), t = 25.0 °C

<table>
<thead>
<tr>
<th>Sample</th>
<th>SAHA (Mixture)</th>
<th>Phen (Mixture)</th>
<th>Phendio (Mixture)</th>
<th>Bipy (Mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LogK</td>
<td>10.50±0.1</td>
<td>4.06±0.1</td>
<td>3.22±0.1</td>
<td>3.28±0.1</td>
</tr>
<tr>
<td>LogK</td>
<td>9.34±0.1</td>
<td>4.94±0.1</td>
<td>-</td>
<td>4.46±0.1</td>
</tr>
</tbody>
</table>

Data presented as estimated protonation constant (±SD)

For the Cu(II)-SAHA system, it was assumed that SAHA coordinated in a typical O,O'-bidentate manner to the metal ion via its hydroxamate moiety.[9] Data from the titration curves were employed to calculate stepwise and overall stability constants (log β); [CuB]⁺ (logβ = 8.2 ± 0.1) and [CuB₂] (logβ = 15.7 ± 0.6), where B = SAHA. Due to the limited solubility of the [CuB₂] system, precipitation occurred during the potentiometric titration at low pH (~5). The stability constant for this system is therefore not truly accurate.

In all N,N'-donor systems studied, complete formation of [CuA]²⁺ (where A = N,N ligand) was observed at the start of each of the titrations with precipitation again at low pH (~5). It was thus not possible to determine overall stability constants for these systems.

2.3.8 Ternary pH potentiometric titrations

Titration of ternary samples of N,N-donor ligand and SAHA were performed at typically 1:1:1, 1:2:1, 1:1:2 & 1:2:2 (Cu:SAHA:N,N'-ligand) ratio. Based on results in section 2.3.7, stability constants were calculated assuming first the formation of [CuA]²⁺ followed by coordination of SAHA. Stability constants (Table 6) were therefore calculated using the following equilibrium process:


\[ [Cu(N,N')] + SAHA \rightleftharpoons [Cu(SAHA)(N,N')] \]

**Table 6:** Calculated stepwise stability constants of ternary Cu-SAHA-N,N ligand systems in 50:50 w/w % (DMSO: H₂O) solution

<table>
<thead>
<tr>
<th>Complex</th>
<th>Log K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-SAHA-Phen</td>
<td>8.64</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>8.91</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>9.30</td>
</tr>
</tbody>
</table>

Stability constant data indicates the formation of [CuAB]⁺ (where A = N,N'-ligand; B = SAHA) is favoured compared to [CuB]⁺ (8.2 ± 0.1). ∆logK values range from 0.44 –1.10 log units which are significantly higher than statistically expected values.[⁸]

\[
\Delta \log K = \log K ([Cu(SAHA-1H)(N,N')]⁺) - \log K ([Cu(SAHA-1H)]⁺)
\]

A trend in stability constants was observed with [Cu(SAHA-1H)(Bipy)]⁺ > [Cu(SAHA-1H)(Phendio)]⁺ > [Cu(SAHA-1H)(Phen)]⁺. Compared to Cu-SAHA-Phen, the higher stability constant of the Cu-SAHA-Phendio may be due to the electron withdrawing nature of the diketone moiety of the Phendio ligand, stabilising the positive charge of the metal centre. The increase in stability constant in the case of the Bipy system may be due to some π stacking interactions between the aromatic rings of Bipy and that of SAHA.

### 2.3.9 Cu-SAHA-DPQ/DPPZ ternary systems

An attempt to measure the stability constants of [CuAB]⁺ systems where A = DPQ or DPPZ and B = SAHA was also undertaken using the experimental conditions outlined in sections 2.2.5 & 2.3.7 – 2.3.8. Similar solubility and precipitation issues were observed at the beginning of the titrations of these systems.
To resolve solubility issues, the DMSO content in the solvent system was increased to 70:30 w/w % (DMSO:H₂O). Despite this increase, stability constants could not be accurately determined for the following reasons.

Despite the increased DMSO content, precipitation and some decomposition of the DPPZ ligand occurred in the basic pH range. The low dielectric constant of the solvent system enhanced the formation of the Cu-SAHA-DPQ complex, which was complete at the beginning of the titrations.

In order to directly compare results for 50:50 and 70:30 w/w % (DMSO:H₂O), parallel potentiometric titrations of the ternary Cu-SAHA-Bipy system in both of these solvent systems were performed. The calculated log $K$ value in 70:30 w/w % (DMSO:H₂O) mixture was 10.66 in contrast to a log $K$ 50:50 w/w % (DMSO:H₂O) of 9.30. The observed increase in stability constant is again thought to be due to the low dielectric constant of this solvent mixture.

2.4 Conclusion

A library of novel Cu(II) complexes incorporating both HDACi and DNA intercalating moieties has been successfully developed. All the complexes were synthesised using a simple, one-step process producing the complexes in good yield and excellent purity. Subsequent chemical characterisation using elemental analyses, ESI-MS, and IR confirmed the successful synthesis of these complexes.

While limited solubility of the complexes in the experimental conditions employed hindered accurate determination of stability constants, stability constants for [Cu(AB)] type systems were determined.

These are the first examples of Cu(II) chemotypes incorporating the clinically used HDACi SAHA and designer intercalating agents, Phen, Phendio, Bipy, DPQ and DPPZ.
2.5 Chapter 2 References


Chapter 3 - *In Vitro* Cytotoxicity, Histone Deacetylase Inhibitory Activity, Anti-Bacterial and Anti-Fungal Properties
3.1. Introduction

The design and development of non-Pt-based metallodrugs as anti-cancer therapeutics is ongoing; the rationale being that if you change the metal, you change the chemistry and thus potentially change the mechanism of action. In so doing, these metallodrugs may overcome some of the drawbacks associated with classical Pt drugs. As stated earlier, there is sound rationale behind the development of Cu complexes as potential anti-cancer agents.

As described in chapter 2, we rationally combined, into one drug molecule, the well-established chemical nuclease activity of Cu-Phen derivatives with the HDACi SAHA generating a library of novel Cu-Phen-SAHA chemotypes. This work was inspired by the anti-cancer properties of HDACi such as SAHA and the chemical nuclease activity of $[\text{Cu(Phen)}_2]^{2+}$ and $[\text{Cu(Phen)}]^2+$ scaffolds. DNA strand scission by $[\text{Cu(Phen)}_2]^{2+}$ arises from its ability to abstract hydrogen from DNA pentose rings in the presence of exogenous reductant (Cu$^{2+}$ → Cu$^+$) and oxidant (O$_2$/H$_2$O$_2$), SAHA, as stated previously, exerts its anti-cancer activity through HDAC inhibition.

![Figure 21 DNA strand scission by Cu-Phen type complexes](image)

We sought to exploit the unique chemical environment of tumour cells by generating bioreductively-activated Cu-Phen-HDACi prodrugs which, upon entry into the reducing environment of tumour cells, would be activated by reduction (Cu(II) to Cu(I)), facilitating both oxidative DNA damage with concomitant release of the HDACi, free to inhibit HDACs. Cu(I) is known to have a very low affinity for hydroxamates.
In order to assess the potential of these complexes to act as anti-cancer agents, their *in vitro* cytotoxic activity was assessed across a panel of cancerous cell lines as well as their ability to inhibit HDAC activity. Their anti-bacterial and anti-fungal activities were also assessed given that many Cu(II) complexes possess these properties. *In vitro* cytotoxicity, HDAC inhibitory activity and anti-bacterial and anti-fungal activities are herein described and discussed.

3.2. Materials and Methods

3.2.1. General chemicals
Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich and used without further purification. Cu-Phen was synthesized using a previously reported method.[1]

3.2.2. Cell Culture
A panel of cancerous cell lines, outlined in Table 7, was used in this study. These cell lines are routinely used for measuring *in vitro* cytotoxicity of novel test compounds. Cells were cultured in a standard 75 cm$^3$ flask (Corning®, Austria) containing RPMI1640 medium (Sigma-Aldrich, Ireland) supplemented with 10% Fetal Bovine Serum (Gibco®, Ireland) at 37 °C in a humidified atmosphere at 5% CO$_2$. Every 3-4 days cells reached 70-80% confluency after which they were harvested with trypsin-EDTA (ATCC, LGC, United Kingdom) and re-suspended in media.
**Table 7** Cell line panel employed in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue, disease type</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-OV-3</td>
<td>Ovarian, Adenocarcinoma</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast, Adenocarcinoma</td>
</tr>
<tr>
<td>DU145</td>
<td>Prostate, carcinoma</td>
</tr>
</tbody>
</table>

### 3.2.3. Guava ViaCount® Assay

Prior to complex addition, SK-OV-3, MCF-7 and DU145 cells were seeded overnight in 96 well tissue culture plates (Costar) at an initial density as outlined in Table 8.

**Table 8** *In vitro* cytotoxicity: Seeding densities (24-72 Hrs) for tested cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>24 Hrs</th>
<th>48 Hrs</th>
<th>72 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-OV-3</td>
<td>$4 \times 10^4$</td>
<td>$2 \times 10^4$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>MCF-7</td>
<td>$2 \times 10^5$</td>
<td>N/A</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>DU145</td>
<td>$2 \times 10^5$</td>
<td>N/A</td>
<td>$1 \times 10^5$</td>
</tr>
</tbody>
</table>

NA – Not Applicable

DMSO stocks of Doxorubicin (Sigma-Aldrich, Ireland) and complexes were prepared at ~ 10 mM. Stock solutions were diluted in supplemented RPMI1640 medium to give the following final concentrations in 200 µl wells: 10, 7.5, 5, 2.5 and 1.25 µM, while Doxorubicin stocks were diluted giving the following final concentrations: 1, 0.75, 0.5, 0.25 and 0.125 µM. A DMSO control of the highest drug incubation was also included. Cells were incubated for 24, 48 and 72 hr at 37 °C in a humidified atmosphere with 5% CO₂. After drug incubation, cells were washed once with 200 µL of phosphate buffered saline (PBS) then harvested with 100 µl 1X trypsin. After
addition of 100 µL media, the cells were transferred to round bottom 96 well plates (Greiner, Austria) containing 10 µl ViaCount® reagent and incubated in the dark at RT for 10 mins prior to analysis. Viability was assessed on a Guava EasyCyte HT flow cytometer using Guava ViaCount® software.

3.2.4. Preparation of nuclear isolates
SK-OV-3 cells were seeded in a 75 cm³ flask and grown until 80-90% confluency. The cells were treated at isotoxic concentrations corresponding to the IC₅₀ value (Table 9) for 24 hr (Cu-SAHA-Phen, Cu-SAHA-Phendio, Cu-SAHA-DPQ, Cu-SAHA-DPPZ, 48 hr (Cu-SAHA-Bipy) or 72 hr (SAHA). Cells were washed twice with ice cold PBS, scraped and collected into a 1.5 ml microcentrifuge tube (Eppendorf, Germany). Nuclear extracts were prepared following the protocol recommended by the manufacturer of the EpiQuik® Nuclear Extraction Kit (Epigentek, USA). Protein concentration of the nuclear isolates was determined by following the protocol of the manufacturer of the Quick Start™ Bradford protein assay (Bio-Rad, CA, USA).

3.2.5. HDAC activity
The SK-OV-3 nuclear extracts were tested for HDAC activity following the protocol recommended by the manufacturer of the EpiQuik® Colorimetric HDAC Activity/Inhibition assay kit (Epigentek, USA). The standard curve was constructed using the standard included in the kit; the absolute amount of deacetylated product was calculated from the standard curve. HDAC activity was calculated by:

$$HDAC\ activity = \frac{OD(\text{Control} - \text{blank}) - OD(\text{Sample} - \text{blank})}{\text{Standard curve slope}}$$

Values in the bar chart were normalised to the control (untreated) sample. 4 µg of protein was added to each sample.
3.2.6. Bacterial cell culture

*In vitro* anti-bacterial activities of test compounds were evaluated by Professor Milan Pour in Charles University, Czech Republic on a panel of three ATCC strains (*Staphylococcus aureus* (SA) ATCC 6538, *Escherichia coli* (EC) ATCC 8739, *Pseudomonas aeruginosa* (PA) ATCC 9027) and five clinical isolates (methicillin resistant *Staphylococcus aureus* (MRSA) HK5996/08, *Staphylococcus epidermidis* (SE) HK6966/08, *Enterococcus sp.* (EF) HK14365/08, *Klebsiella pneumoniae* (KP) HK11750/08, *Klebsiella pneumoniae* ESBL (KPE) HK14368/08) from the collection of bacterial strains deposited at the Department of Biological and Medical Sciences, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic. The above-mentioned ATCC strains also served as the quality control strains. All the isolates were maintained on Mueller–Hinton agar prior to being tested.

Minimum inhibitory concentrations (MICs) were determined by a modified Clinical and Laboratory Standards Institute (CLSI) standard of microdilution format of the M07-A9 and M07-A8 documents.[2] DMSO (100%) served as a diluent for all compounds; the final concentration did not exceed 2%. Mueller–Hinton agar (MH, HiMedia, Câdersky-Envitek, Czech Republic) buffered to pH 7.4 (±0.2) was used as the test medium. The wells of the microdilution tray contained 200 μl of the Mueller–Hinton medium with 2-fold serial dilutions of the compounds (1000–0.244 μmol/l) and 10 μl of inoculum suspension. Inoculum in MH medium was prepared to give a final concentration of 0.5 McFarland scale (1.5 × 10⁸ cfu·ml⁻¹). The trays were incubated at 37 °C, and MICs were read visually after 24 and 48 hr. The MICs were defined as 95% inhibition of the control growth. MICs were determined twice and in duplicate. The deviations from the usually obtained values were no higher than the nearest concentration value up and down the dilution scale.
3.2.7. Fungal cell culture

*In vitro* anti-fungal activities of the compounds were evaluated by Professor Milan Pour in Charles University, Czech Republic on a panel of four ATCC strains (*Candida albicans* ATCC 44859 (CA1); *Candida albicans* ATCC 90028 (CA2); *Candida parapsilosis* ATCC 22019 (CA3); *Candida krusei* ATCC 6258 (CK1)) and eight clinical yeast isolates (*Candida krusei* E28 (CK2); *Candida tropicalis* 156 (CT); *Candida glabrata* 20/l (CG); *Candida lusitaniae* 2446/l (CL); *Trichosporon asahii* 1188 (TA)) and filamentous fungi (*Aspergillus fumigatus* 231 (AF); *Absidia corymbifera* 272 (AC); *Trichophyton mentagrophytes* 445 (TM)) from the collection of fungal strains deposited at the Department of Biological and Medical Sciences, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic. Three ATCC strains were used as the quality control strains. All of the isolates were maintained on Sabouraud dextrose agar prior to being tested.

Minimum inhibitory concentrations (MICs) were determined by modified CLSI standard of microdilution format of the M27-A3 and M38-A2 documents.[3] DMSO (100%) served as a diluent for all compounds; the final concentration did not exceed 2%. RPMI 1640 (Sevapharma, Prague) medium supplemented with *L*-glutamine and buffered with 0.165 M morpholinepropanesulfonic acid (Serva) to pH 7.0 by 10 M NaOH was used as the test medium. The wells of the microdilution tray contained 200 μL of the RPMI 1640 medium with 2-fold serial dilutions of the compounds (1000 – 0.244 μmol/L for the new compounds) and 10 μL of inoculum suspension. Fungal inoculum in RPMI 1640 was prepared to give a final concentration of 5 × 103 ± 0.2 cfu·mL⁻¹. The trays were incubated at 35 °C, and MICs were read visually after 24 and 48 hr. The MIC values for the dermatophytic strain (*T. mentagrophytes*) were determined after 72 and 120 hr. The MICs were defined as 80% inhibition (IC₈₀) of the control growth for yeasts and as 50% inhibition (IC₅₀) of the control growth for filamentous fungi. MICs were determined twice and in duplicate. The deviations from the usually obtained values were no higher than the nearest concentration value up and down the dilution scale.
3.3. Results & Discussion

3.3.1. In Vitro Cytotoxicity

To investigate the in vitro cytotoxic properties of the Cu(II)-Phen-SAHA chemotypes, complex concentrations which induced 50% inhibition of cellular proliferation (IC$_{50}$) across a panel of 3 cancerous cell lines at 24, 48 and 72 hr time points were first determined via a flow cytometric assay, Guava ViaCount®. The ViaCount® assay principle is based on differential membrane permeability of two fluorescent DNA intercalators of which live and dead cell ratios can be measured. Sigmoidal dose-response curves were plotted and concentrations which inhibit 50% cellular proliferation (IC$_{50}$ values) were determined via statistical analysis at the 95% confidence interval.

The in vitro cytotoxic activity of the Cu(II)-Phen-SAHA chemotypes was first assessed on the human ovarian adenocarcinoma cell line, SK-OV-3. This cell line was chosen on the premise that SK-OV-3 cells possess both a mutant p53 gene and are also intrinsically resistant to cisplatin.$^{[4]}$ ($^\text{Table 9}$). As a representative control, doxorubicin (US trade name Adriamycin) was selected due to its known DNA-damaging properties and its ability to inhibit topoisomerase II.$^{[5]}$ After 24 hr drug treatment, the complexes showed significant in vitro cytotoxicity with low µM IC$_{50}$ values (< 5 µM) observed. The order of activity after 24 hr exposure was Cu-SAHA-DPPZ (1.31 ± 0.27) > Cu-SAHA-Phendio (2.29 ± 0.16) > Cu-SAHA-DPQ (2.64 ± 0.17) > Cu-SAHA-Phen complex (4.54 ± 0.20). Following post 24 hr drug exposure, a shift in IC$_{50}$ from higher to lower values was observed for all complexes tested. After 48 hr the Cu-SAHA-Phendio and Cu-SAHA-DPPZ complexes showed nanomolar in vitro cytotoxic activity (0.77 ± 0.10 µM & 0.70 ± 0.17 µM respectively). Interestingly, the Cu-SAHA-DPPZ chemotype was found to have an IC$_{50}$ value after 24 hr of 1.31 ± 0.27 µM on par with that for SAHA (1.60 ± 0.17 µM) after 72 hr.
Table 9 In vitro cytotoxicity (IC_{50} values) for SK-OV-3 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 Hrs</th>
<th>48 Hrs</th>
<th>72 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.77 ± 0.08</td>
<td>0.76 ± 0.16</td>
<td>&lt;1.25</td>
</tr>
<tr>
<td>SAHA</td>
<td>&gt;10</td>
<td>1.76 ± 0.32</td>
<td>1.60 ± 0.17</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>&gt;10</td>
<td>2.14 ± 0.24</td>
<td>2.45 ± 0.14</td>
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<tr>
<td>Cu-SAHA-Phen</td>
<td>4.54 ± 0.20</td>
<td>2.32 ± 0.18</td>
<td>1.14 ± 0.10</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ</td>
<td>2.64 ± 0.17</td>
<td>1.60 ± 0.20</td>
<td>1.60 ± 0.20</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>1.31 ± 0.27</td>
<td>0.70 ± 0.17</td>
<td>0.74 ± 0.20</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>2.29 ± 0.16</td>
<td>0.77 ± 0.10</td>
<td>&lt;1.25</td>
</tr>
</tbody>
</table>

Data points representative of an average of triplicate measurements, IC_{50} (µM) ± SD, N=3

The in vitro cytotoxicity of the complexes were further evaluated against two additional cancerous cell lines: DU145 (prostate) and MCF-7 (breast) at 24 and 72 hr time points. DU145 cells were chosen to not only broaden the panel of cancer cell lines being tested but also because they, like SK-OV-3, possess a mutant p53 gene.[6] MCF-7 was chosen, again to further broaden the panel of cancer cell lines being tested. They have also been found to be slow-growing as well as being poorly responsive to some Cu complexes described in the literature.[7] After 24 hr of treatment, the complexes demonstrated significant in vitro cytotoxicity in the DU145 prostate cells (Table 10) with Cu-Phendio-SABA being the most potent with an IC_{50} of 0.09 ± 0.52 µM. The IC_{50} values for the Cu-SAHA-DPQ and Cu-SAHA-DPPZ complexes, whilst being highly cytotoxic, deviated little between 24 and 72 hr. Complexes had on par or more enhanced cytotoxicity as compared to SAHA. The overall IC_{50} trend for this prostate cell line is Cu-SAHA-Phendio >> Cu-SAHA-DPQ > Cu-SAHA-Phen > Cu-SAHA-Bipy > Cu-SAHA-DPPZ. Nanomolar activity was also observed for Cu-SAHA-DPQ and Cu-SAHA-Phendio after 72 hours (0.95 ± 0.08 and <1.25 µM respectively).
Table 10 *In vitro* cytotoxicity (IC$_{50}$ values) for DU145 and MCF-7 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 Hrs IC$_{50}$ (µM)</th>
<th>72 Hrs IC$_{50}$ (µM)</th>
<th>24 Hrs IC$_{50}$ (µM)</th>
<th>72 Hrs IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Doxorubicin</em></td>
<td>0.15 ± 0.02</td>
<td>&lt;1.25</td>
<td>1.44 ± 0.28</td>
<td>0.94 ± 0.19</td>
</tr>
<tr>
<td>SAHA</td>
<td>&gt;10</td>
<td>1.99 ± 0.26</td>
<td>&gt;10</td>
<td>8.82 ± 1.13</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>&gt;10</td>
<td>2.29 ± 0.18</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Cu-SAHA-Phen</td>
<td>2.64 ± 1.16</td>
<td>1.94 ± 0.20</td>
<td>&gt;10</td>
<td>4.82 ± 0.32</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>3.43 ± 0.31</td>
<td>3.51 ± 0.20</td>
<td>2.68 ± 0.26</td>
<td>4.89 ± 0.19</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>0.09 ± 0.52</td>
<td>&lt;1.25</td>
<td>1.20 ± 0.16</td>
<td>0.72 ± 0.32</td>
</tr>
</tbody>
</table>

Data points representative of an average of three independent measurements, IC$_{50}$ (µM) ± SD, N=3

All complexes tested demonstrated potent cytotoxicity also against the MCF-7 breast cancer cells with the Cu-Phendio-SAHA being the most cytotoxic agent with an IC$_{50}$ in the nM range. Compared to SAHA alone, Cu-SAHA-DPPZ, Cu-SAHA-Phen and Cu-SAHA-Phendio have enhanced cytotoxicity against the MCF-7 cells. It has been reported in the literature that SAHA possesses preferential cytotoxicity to mutant p53 cancerous cells.\[8\] These results support these data in that SAHA has shown preferential cytotoxicity towards DU145 and SK-OV-3 cells while being less active in MCF-7 cells. Interestingly, this effect is conserved in the Cu(II) complexes where no cytotoxic activity for both Cu-SAHA-Bipy and Cu-SAHA-DPQ was observed. This initial observation may indicate that the Cu(II) chemotypes investigated may be selective towards ovarian and prostate cancerous cell lines harbouring mutant p53 genes.

3.3.2. HDAC inhibitory activity

As mentioned in chapter 2, the Cu(II) chemotypes were rationally designed as dual functioning agents. We hypothesized that, upon entry into cancerous cells, the redox-active Cu(II) ion would be reduced to Cu(I) with concomitant
release of the HDACi (SAHA) and the chemical nuclease moiety (Cu-N,N'-ligand). To investigate the HDAC inhibition arm of this hypothesis, the HDAC inhibitory properties of the complex series were investigated utilising a commercially available HDAC activity enzyme linked immunosorbent assay (ELISA) kit. The HDAC activity ELISA kit directly measures the amount of an immobilised acetylated histone substrate after incubation with sample(s) containing HDAC protein. During this assay, a sample containing HDAC enzymes is added to the sample wells containing an immobilised acetylated histone protein substrate. HDAC enzymes bind to this substrate and convert it to the deacetylated form. After incubation with various antibodies, wash steps and addition of reagents, a colour change can be monitored correlating with HDAC function. Following treatment of SK-OV-3 cells with SAHA after 72 hrs, nuclear extracts containing HDAC enzymes were isolated and their HDAC activity measured using this ELISA kit. If treatment of the Cu(II) complexes, for example, releases SAHA into the cellular environment and inhibits HDAC function this, in turn, will result in substrate remaining in the acetylated form (i.e. an increase in colour change and vice versa).

![Figure 22 HDAC inhibitory activity of Cu(II) complexes](image)

*Figure 22 HDAC inhibitory activity of Cu(II) complexes:* SK-OV-3 cells were incubated with Cu complexes and SAHA corresponding to the IC\(_{50}\) value for 24 hr (Cu-SAHA-Phen; Cu-SAHA-Phendione; Cu-SAHA-DPQ; Cu-SAHA-DPPZ), 48 hr (Cu-SAHA-Bipy) or 72 hr (SAHA). Nuclear isolates were isolated according to the protocol recommended by the manufacturer. SK-OV-3 nuclear extracts were tested for their HDAC activity according to the protocol recommended by the manufacturer (Epigentek, USA). Data points representative of an average of triplicate measurements, N=3.
As outlined in section 3.2.4, SK-OV-3 cells were treated at isotoxic concentrations of the test complexes corresponding to their IC$_{50}$ values at 24, 48 or 72 hr. After appropriate treatment with test agents the nuclear fraction of SK-OV-3 cells was isolated and HDAC activity directly measured. As expected, the HDAC activity of SAHA treated cells was significantly reduced compared to control (non-drug treated cells) where a ~90% reduction in HDAC activity was observed after 72 hr drug treatment. The Cu(II) chemotypes significantly inhibited HDAC function after 24 hr and 48 hr drug treatment. Interestingly, Cu-SAHA-DPPZ and Cu-SAHA-Phen had on par HDAC activity after only 24 hr compared to SAHA after 72 h drug treatment at isotoxic concentrations. This could potentially be due to greater cellular accumulation of the complexes relative to SAHA with subsequent Cu(II) to Cu(I) reduction and SAHA release free to inhibit HDACs or that the complexes themselves have more potent HDAC inhibitory activity or that the complexes induce epigenetic changes within SK-OV-3 cells. Cu-SAHA-DPQ, Cu-SAHA-Phendio and Cu-SAHA-Bipy had comparable HDAC activity while being slightly less efficient as compared to SAHA at 72 hr drug treatment.

3.3.3. In Vitro Anti-Bacterial and Anti-Fungal Activity

As stated earlier, the anti-bacterial and anti-fungal activities of the Cu(II) chemotypes were also assessed given that many Cu(II) complexes possess these properties. Specifically, the anti-microbial and anti-fungal activity of Cu(II) salts, test complexes and ligands were tested at 24 and 48 hr intervals across an extensive panel of gram positive bacteria (*Staphylococcus aureus* (SA); methicillin resistant *Staphylococcus aureus* (MRSA); and *Staphylococcus epidermidis* (SE)), gram negative bacteria (*Enterococcus* sp. (EF); *Escherichia coli* (EC); *Klebsiella pneumonia* (KP); *Klebsiella pneumonia* (KP-E) & *Pseudomonas aeruginosa* (PA)), non-filamentous fungi: (*Candida albicans* ATCC 44859 (CA1); *Candida albicans* ATCC 90028 (CA2); *Candida parapsilosis* ATCC 22019 (CA3); *Candida krusei* ATCC 6258 (CK1); and clinical yeast isolates (*Candida krusei* E28 (CK2); *Candida tropicalis* 156 (CT); CG, *Candida glabrata* 20/I (CG); *Candida lusitaniae* 2446/I (CL); *Trichosporon asahii* 1188 (TA), filamentous fungi *Aspergillus fumigatus* 231
(AF); *Absidia corymbifera* 272 (AC) & *Trichophyton mentagrophytes* 445 (TM)).

No anti-microbial activity (Table 11, Figure 23) was observed across all tested strains for SAHA with a minimum inhibitory concentration 95% (MIC$_{95}$) values > 2,000 µM. Likewise, no significant activity was observed for the $N,N'$-ligands with the exception of the DPQ and DPPZ ligands, which had MIC$_{95}$ values ranging from 3.90 – 125 µM with particular activity observed in the SA, MRSA, SE & EF strains. The most active complexes in the series were Cu-SAHA-DPPZ and Cu-SAHA-DPQ with MIC$_{95}$ ranging from 7.81-125 µM across the tested strains. It would seem that both of these complexes target gram positive bacteria (SA, MRSA & SE) selectively where lower activity (7.81-31.25 µM) was observed compared to gram negative strains (7.81 – 1,000 µM).
Table 11 Tabulated MIC<sub>95</sub> (µM) values (Bacteria, 48 Hrs): Anti-microbial activity (MIC<sub>95</sub>) of Cu(II) salts, test complexes and ligands following 48 h drug exposure (24 and 48 hour values were identical). SA - *Staphylococcus aureus*; MRSA - methicillin resistant *Staphylococcus aureus*; SE - *Staphylococcus epidermidis* EF - *Enterococcus* sp.; EC - *Escherichia coli*; KP - *Klebsiella pneumoniae*; KP-E - *Klebsiella pneumoniae*; PA - *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Gram positive</th>
<th>Gram Negative</th>
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<tr>
<td></td>
<td>SA</td>
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<tr>
<td>Phen</td>
<td>125</td>
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</tr>
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<td>DPQ</td>
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<td>DPPZ</td>
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<td>Cu-Phen</td>
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<tr>
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</table>

¹Cu<sup>2+</sup> = CuCl<sub>2</sub>
Figure 23 *In vitro* anti-bacterial activity: Graphic representation of MIC$_{95}$ of Cu(II) salt, test complexes and ligands across a panel of bacteria following 48 hour drug exposure (24 and 48 h MIC$_{95}$ values were identical). SA - *Staphylococcus aureus*; MRSA - methicillin resistant *Staphylococcus aureus*; SE - *Staphylococcus epidermidis*; EF - *Enterococcus* sp.; EC - *Escherichia coli*; KP - *Klebsiella pneumonia*; KP-E - *Klebsiella pneumonia*; PA - *Pseudomonas aeruginosa*
In contrast to the anti-bacterial properties, the in vitro anti-fungal activity (Table 12, Figure 24) of the metal-free Phen ligand surpassed that of all of the complexes tested, with consistent MIC$_{95}$ ranging from 7.81 – 15.62 µM in all fungal cell lines tested. This observation is in agreement with previously published results$^{[9]}$ most likely due to the sequestering properties of this ligand for trace metal ions (e.g. Zn$^{2+}$) which are essential for fungal survival. Cu-Phen exhibited broad spectrum anti-fungal activity across the panel tested. Cu-SAHA-DPPZ was found to have enhanced activity towards the non-filamentous CK1 and CK2 strains with consistent MIC$_{80}$ values of 7.81 µM. Little activity was observed for this complex across the panel tested except in the case of TM where an MIC$_{80}$ of 31.25 µM was observed. All other complexes showed little anti-fungal activity towards the panel tested, with the exception of Cu-SAHA-DPQ which had an MIC$_{80}$ of 62.5 µM for the CK1 strain.
Table 12 Tabulated MIC	extsubscript{50} values (µM) values (Fungi, 48 Hrs): Anti-fungal activity (MIC\textsubscript{50}) of Cu(II) salts, test complexes and ligands following 48 h drug exposure (24 and 48 h values were identical). CA1 - Candida albicans ATCC 44859; CA2 - Candida albicans ATCC 90028 (CA2); CA3 - Candida parapsilosis ATCC 22019; CK1 - Candida krusei ATCC 6258; CK2 - Candida krusei E28; CT - Candida tropicalis 156; CG - Candida glabrata 20/I; CL - Candida lusitaniae 2446/I; TA - Trichosporon asahii 1188; AF - Aspergillus fumigatus 231; AC - Absidia corymbifera 272; TM - Trichophyton mentagrophytes 445

<table>
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<tr>
<th>Compound</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>CK1</th>
<th>CK2</th>
<th>CT</th>
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<td>31.25</td>
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</tbody>
</table>

\textsuperscript{1}Cu\textsuperscript{2+} = CuCl\textsubscript{2}
**Figure 24** *In vitro* anti-fungal activity: Graphic representation of MIC\textsubscript{80} of Cu\textsuperscript{2+} salt, test complexes and ligands across a panel of bacteria following 48 h drug exposure (24 and 48 h MIC\textsubscript{80} values were identical). CA1 - *Candida albicans* ATCC 44859; CA2 - *Candida albicans* ATCC 90028 (CA2); CA3 - *Candida parapsilosis* ATCC 22019; CK1 - *Candida krusei* ATCC 6258; CK2 - *Candida krusei* E28; CT - *Candida tropicalis* 156; CG - *Candida glabrata* 20/I; CL - *Candida lusitaniae* 2446/I; TA - *Trichosporon asahii* 1188; AF - *Aspergillus fumigatus* 231; AC - *Absidia corymbifera* 272; TM - *Trichophyton mentagrophytes* 445
3.4. Conclusion

As described in chapter 2, we rationally designed a series of complexes for potential use as anti-cancer agents. We have shown that these complexes, in particular Cu-SAHA-Phendio, Cu-SAHA-DPQ and Cu-SAHA-DPPZ have both promising μM & nM in vitro cytotoxic activity against both ovarian (SK-OV-3) and prostate (DU145) cancerous cells. We also investigated both the in vitro anti-bacterial and anti-fungal activity across a large panel of pathogenic microbes; we found Cu-SAHA-DPQ and Cu-SAHA-DPPZ had activity particularly towards Gram positive bacteria (7.81 – 31.25 µM). We also found Cu-SAHA-DPPZ to be active against two non-filamentous fungal strains (CK1 & CK2, 7.81 µM) and one filamentous strain (TM, 31.25µM).

We also investigated the HDAC inhibitory properties of the Cu(II) chemotypes in SK-OV-3 cells; all the Cu(II) complexes were capable of inhibiting HDAC function at a shorter time interval compared to the clinical agent, SAHA. It has been well documented in the literature that hydroxamates are subject to in vivo metabolic degradation resulting in shorter half-lives; \(^{[10]}\) a limitation often associated with the clinical development of hydroxamate-based drugs. As the hydroxamate functionality is directly coordinated to the Cu(II) in the Cu chemotypes described, the Cu(II) ion can essentially protect the HDACi hydroxamate functionality until such time as the chemotype reaches its biological target whereupon the HDACi is released following reduction from Cu(II) to Cu(I), ultimately potentially improving the pharmacokinetic profile of hydroxamate-based HDAC inhibitors. An added advantage therefore of the complexes described herein is that coordinating HDACi with metal cations such as Cu(II) may well widen their therapeutic utility.
3.5. Chapter 3 References


Chapter 4 - Copper-SAHA-\(N,N'\)-Chemotypes and Their Interactions with Nucleic Acids
4.1 Introduction

Having established that the Cu-SAHA-\(N,N'\)-chemotypes had potent \textit{in vitro} cytotoxicity as well as HDAC inhibitory activity as described in chapter 3, we sought to understand better their interactions with nucleic acids and to gain a deeper insight into their overall mechanism of action. As previously outlined in chapter 1, Cu complexes containing intercalating ligands have been shown to interact with DNA via intercalation between DNA bases and/or non-covalent major or minor groove binding. We sought to establish the DNA binding profile of these chemotypes through competitive EtBr displacement, viscosity measurements, fluorescence quenching and thermal melting analysis experiments. Cu(II) complexes, because of their redox active nature, have also been shown to cause oxidative DNA damage through the generation of ROS species following reduction from Cu(II) to Cu(I). We also therefore wished to establish whether these chemotypes generated ROS species and, if so, which species, if any, were predominant. Herein we describe the DNA binding and chemical nuclease activity of these Cu-SAHA-\(N,N'\)-chemotypes.

4.2 Materials and Methods

4.2.1. General chemicals

poly[\(d(G-C)_{2}\)] (P9389, \(\epsilon_{260} = 16,800 \text{ M(bp)}^{-1} \text{ cm}^{-1}\)), poly[\(d(A-T)_{2}\)] (P0833, \(\epsilon_{260} = 16,800 \text{ M(bp)}^{-1} \text{ cm}^{-1}\)), ethidium bromide (EtBr), netropsin (N9653), Actinomycin D (A-1410), \(N,N'\)-Dimethylformamide (DMF, 22705-6) and salmon testes DNA (stDNA, D1626- 1G) were purchased from Sigma-Aldrich and used without further purification. pUC19 vector (New England Bio-Labs, N3041), UltraPure calf thymus DNA (ctDNA, Invitrogen, 15633-019, \(\epsilon_{260} = 12.824 \text{ M(bp)}^{-1} \text{ cm}^{-1}\)), sodium chloride (NaCl, Ambion, AM9760G) and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (Fisher, 10041703) were purchased from respective suppliers and used without further purification.
4.2.2. Competitive EtBr displacement

Competitive EtBr displacement assays were conducted using a method previously reported by Kellett et al.[1] Briefly, a working solution of 20 µM ctDNA, poly[d(G-C)]₂ or poly[d(A-T)]₂; 25.2 µM EtBr; 40 mM NaCl in HEPES buffer (80 mM, pH 7.2) was prepared. Stock solutions of metal complexes, SAHA and groove binding drugs were prepared in N,N'-Dimethyl formamide (DMF) at ~10 mM and further diluted with 80 mM HEPES. 50 µl of DNA-Et working solution was placed into each well of a 96 well microplate, with the exception of blanks which contained 100 µl of HEPES buffer. Serial aliquots of the metal complexes, SAHA and groove binding drugs were added to the working solution and the final volume was adjusted to 100 µl in each well such that the final concentrations of ctDNA and EtBr were 10 and 12.6 µM, respectively. The plate was incubated at room temperature for 1 hour, protected from light. Microplates were analysed using a Bio-Tek synergy HT multi-mode microplate reader with excitation and emission wavelengths set to 530 and 590 nm respectively. Each drug concentration was measured in triplicate and the apparent binding constants were calculated using $K_{app} = K_e \times 12.6/C_{50}$, where $K_e = 9.5 \times 10^6$ M(bp)$^{-1}$.

4.2.3 Viscosity measurements

A 15 ml solution of stDNA was prepared at $1 \times 10^{-3}$ M in 80 mM HEPES buffer for each working sample. Stock solutions prepared in DMF were added according to the gradual increasing [drug]/[DNA] (r) ratios of 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18 and 0.2. Viscosity values, $\eta$, (unit: cP) were directly obtained by running 0# spindle in working samples at 60 rpm via DV-II-Programmable Digital Viscometer equipped with Enhanced Brookfield UL Adapter at room temperature. Data were presented as $\eta / \eta_0$ versus [compound]/[DNA] ratio, in which $\eta_0$ and $\eta$ refers to viscosity of each DNA working sample in the absence and presence of complex[1].
4.2.4 Fluorescence quenching

A working solution of 50 µM UltraPure ctDNA ($\varepsilon_{260} = 12,824 \text{ M (bp)}^{-1} \text{ cm}^{-1}$) along with either 10 µM EtBr or Hoechst 33258 (Sigma) in HEPES buffer (80 mM, pH = 7.2) and NaCl (40 mM) was prepared. Stock solutions of metal complexes, metal salts, free ligands and groove binding drugs were prepared at ~4.0 mM in DMSO and diluted further with 80 mM HEPES. 50 µL of DNA-EtBr or DNA-Hoechst working solutions were placed in each well of a 96 well microplate with the exception of the blanks which contained 100 µL HEPES buffer and 5 µM of either Hoechst or EtBr. Serial aliquots of the test compound were added to the working solutions and the volume was adjusted to 100 µL in each well such that the final concentrations of ctDNA and EtBr/Hoechst were 25 µM and 5 µM, respectively. The plate was allowed to incubate at room temperature for 5 minutes before being analysed using a Bio-Tek synergy HT multi-mode microplate reader with excitation and emission wavelengths being set to 530 and 590 nm for EtBr detection or 360 nm and 460 nm for Hoechst 33258 detection. Concentrations of the tested compounds were optimized such that fluorescence was 30-40% of the initial control at their highest reading. Each drug concentration was measured in triplicate, on at least two separate occasions. From a plot of fluorescence versus added drug concentration, the $Q$ value is given by the concentration required to effect 50% removal of the initial fluorescence of bound dye.\[1\]

4.2.5. Thermal melting

Analysis was carried out on an Agilent Cary 100 dual beam spectrophotometer equipped with a 6 × 6 Peltier multicell system with temperature controller. For poly[d(G-C)$_2$]; in a final volume of 1 ml using Starna black-walled quartz cuvettes with tight-fitting seals, 2 mM NaOAc buffer (pH = 5.0), 1 mM NaCl and poly[d(G-C)$_2$] (Sigma, P9389) were added to give a final absorbance of between 0.18 and 0.20 absorbance units at 260 nm ($\varepsilon_{\text{max}} = 8400 \text{ M}^{-1} \text{ cm}^{-1}$). For poly[d(A-T)$_2$]; in a final volume of 1 ml using Starna black-walled quartz cuvettes with tight-fitting seals, 50 mM NaOAc buffer (pH = 5.0), 250 mM NaCl and poly[d(A-T)$_2$] (Sigma, PO883) were
added to give a final absorbance of between 0.18 and 0.20 absorbance units at 260 nm ($\varepsilon_{\text{max}} = 6600 \text{ M}^{-1} \text{ cm}^{-1}$). Stock solutions of metal complexes, netropsin, and actinomycin D, prepared beforehand in DMF, were further diluted in 80 mM HEPES (pH 7.2). An aliquot of test reagent was then added to each cuvette such that an $r$ value of 0.1 was achieved ($r = [\text{compound}]/[\text{nucleotide}]$). The test reagent and respective alternating copolymer were then incubated for 10 min at 20 °C prior to commencing the temperature ramp. Thermal melting measurements were recorded at 260 nm at 0.25 s intervals. Temperature was ramped at 3 °C/min over the range of 20.0–97.0 °C. The spectral bandwidth (SBW) was set to 1. Temperature was calibrated, for each measurement, using a temperature probe placed in an identical black-walled cuvette containing equivalent buffer and NaCl concentrations. Samples were run in triplicate, and the melting temperature $T_M$ (°C) was calculated using the built-in derivative method on the instrument[2].

4.2.6 Nuclease activity in the presence or absence of ROS scavengers

The presence or absence of ROS specific scavengers were used to determine the effect on the DNA cleavage abilities of each Cu complex. The procedure was adapted from a previously reported method.[3] Briefly, in a final volume of 20 µl, 80 mM HEPES (pH = 7.2), 25 mM NaCl, 1 mM Na-L-ascorbate, and 400 ng of pUC19 DNA treated with drug concentrations of 1, 2.5, 3.75 and 5 µM (Cu-SAHA-Phen, Cu-SAHA-DPQ and Cu-SAHA-Phendio), 2.5, 5, 10, 15 µM (Cu-SAHA-Bipy), 1, 2.5, 5, 10 µM (Cu-SAHA-DPPZ) and 250 nM, 500 nM 1 µM and 2.5 µM (Cu-Phen) in the presence or absence of ROS scavengers/stabilisers: KI (10 mM), NaN$_3$ (10 mM), DMSO (10%) and Tiron (10 mM). Reactions were incubated for 30 minutes at 37 °C and quenched with 6X loading dye (Fermentas) containing 10mM Tris-HCl, 0.03% bromophenol blue, 0.03% xylene cyanole FF, 60% glycerol and 60 mM EDTA. Samples were then loaded onto an agarose gel (1.2%) containing 4 µl of EtBr. Electrophoresis was completed at 70 V for 2 hrs in 1X TAE buffer.
4.3 Results & Discussion

4.3.1. DNA binding affinity and intercalative studies

In order to investigate the apparent binding constants ($K_{\text{app}}$) or binding affinity of the complex series towards canonical double stranded (dsDNA) – ctDNA, a high throughput competitive EtBr displacement assay was used. A solution of ctDNA was treated with an excess of EtBr resulting in a highly fluorescent DNA sample. Titration of the test complex at various concentrations results in DNA binding interactions such as intercalation, and thus ejection of the bound EtBr to the DNA backbone and a reduction in fluorescence. $K_{\text{app}}$ values can be calculated as outlined in section 4.2.2 and Table 13. Actinomycin D$^4$ and netropsin$^5$ were used throughout the study as reference standards due to their known intercalative and minor groove binding properties, respectively. The presence of the phenazine ligands DPQ and DPPZ in the coordination environment around the Cu(II) metal centre were found to significantly enhance DNA binding with calculated $K_{\text{app}}$ values ($7.13 \times 10^6$ M(bp$^{-1}$) and $9.0 \times 10^6$ M(bp$^{-1}$) respectively) exhibiting a significant enhancement compared to the Cu-SAHA-Phen derivative ($9.65 \times 10^5$ M(bp$^{-1}$)).

Furthermore we sought to investigate the intercalative effects of the complex series on salmon testes DNA (stDNA) using viscosity measurements. Introduction of an intercalative agent such as actinomycin D results in an increase in relative viscosity due to conformational changes induced after intercalation between the DNA base pairs accordingly, while introduction of DNA surface-binding species such as netropsin and [Co(NH$_3$)$_6$]Cl$_3$ induces a moderate or diminished effect (Figure 25 and Table 13). Cu-SAHA-DPPZ and Cu-SAHA-DPQ produced an enhanced viscosity profile with Cu-SAHA-DPPZ showing intercalative activity equivalent to the known DNA intercalator actinomycin D. Interestingly, the Cu-SAHA-Phendio and Cu-SAHA-Bipy complexes demonstrated only moderate intercalative effects which are in agreement with observations for the $K_{\text{app}}$ measurements. Overall, a trend in intercalative activity was observed in the Cu(II) chemotypes where: Cu-
SAHA-DPPZ>Cu-SAHA-DPQ>>Cu-SAHA-Phen>>Cu-SAHA-Phendio>Cu-SAHA-Bipy.

Figure 25 Competitive EtBr displacement and viscosity analysis: Competitive EtBr displacement assays with ctDNA and Viscosity measurements with stDNA. Data points are presented as an average of triplicate measurements ± SD. Data points for SAHA are emitted for clarity.

Table 13 (A) Apparent binding constants ($K_{app}$) determined by competitive EtBr displacement measurements using ctDNA (B) relative viscosity at $r=0.18$ using stDNA

<table>
<thead>
<tr>
<th>Complex</th>
<th>$C_{50}$ ($\mu$M)</th>
<th>$K_{app}$ $M$(bp)$^{-1}$</th>
<th>$\eta/\eta_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>04.10</td>
<td>$2.92 \times 10^7$</td>
<td>1.14</td>
</tr>
<tr>
<td>Netropsin</td>
<td>46.27</td>
<td>$2.50 \times 10^6$</td>
<td>1.00</td>
</tr>
<tr>
<td>[Co(NH$_3$)$_6$]Cl$_3$</td>
<td>&gt;300</td>
<td>NC</td>
<td>0.82</td>
</tr>
<tr>
<td>Cu-Phen$_{13j}$</td>
<td>179.21</td>
<td>$6.67 \times 10^5$</td>
<td>1.17</td>
</tr>
<tr>
<td>Cu-SAHA-Phen</td>
<td>124.00</td>
<td>$9.65 \times 10^5$</td>
<td>1.05</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ</td>
<td>16.80</td>
<td>$7.13 \times 10^6$</td>
<td>1.14</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>13.30</td>
<td>$9.00 \times 10^6$</td>
<td>1.07</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>&gt; 150</td>
<td>NC</td>
<td>1.01</td>
</tr>
<tr>
<td>Cu-SHA-Bipy</td>
<td>&gt; 150</td>
<td>NC</td>
<td>1.03</td>
</tr>
<tr>
<td>SAHA</td>
<td>&gt; 150</td>
<td>NC</td>
<td>0.98</td>
</tr>
</tbody>
</table>

$^a$Concentration required to reduce fluorescence by 50%. $^b$Apparent binding constant ($K_{app} = K_e \times C_{50}$, where $K_e = 9.5 \times 10^6$ M(bp)$^{-1}$). $^c$Relative viscosity at $r = 0.18$. drug load of 0.18%. NC = Not Calculated
4.3.2. Fluorescence quenching

In order to identify potential binding specificity, fluorescence quenching experiments of limited bound ctDNA solutions of EtBr (intercalator) and Hoechst 33258 (minor groove binder) were employed (Figure 26 and Table 14). Fluorescence quenching utilises an unsaturated dye-DNA solution in which a limited amount of binding sites are occupied by the appropriate dye. Introduction of the test complex to the DNA solution may cause displacement either directly or indirectly (i.e. conformational changes resulting in dye ejection) and can provide an insight into binding preference (minor groove versus intercalation). In the case of the minor groove binding agent netropsin, a high fluorescence quenching affinity ($Q$) for Hoechst 33258 bound DNA was observed; unsurprisingly the intercalating agent actinomycin D, had a much lower $Q$ value towards the same dye-DNA solution, but was more specific toward EtBr-bound ctDNA. The complex series did not exhibit a large degree of discrimination for quenching either EtBr or Hoechst with the Cu-SAHA-DPPZ and Cu-SAHA-DPQ complexes having the highest $Q$ values of the series and also being higher than the well-studied chemical nuclease, Cu-Phen. However, both of these complexes displace Hoechst with slight preference over EtBr whereas with Cu-Phen, this effect is essentially reversed. As expected, no significant quenching was observed for both the Cu-SAHA-Bipy and Cu-SAHA-Phendio at concentrations >150 µM.

![Figure 26](image_url)

**Figure 26** Competitive EtBr displacement and viscosity analysis: Fluorescence quenching of limited bound EtBr intercalator (left) and Hoechst 33258 (right) displacement assays with ctDNA and Viscosity measurements with stDNA. Data points are presented as an average of triplicate measurements ± SD. Data points for SAHA are emitted for clarity.
Table 14 Fluorescence Quenching ($Q^a$) values obtained against ctDNA bound with Hoechst 33258 (groove binder) or EtBr (intercalator).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$Q^a$ (EtBr, µM)</th>
<th>$Q^a$ (Hoescht, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>04.78</td>
<td>26.34</td>
</tr>
<tr>
<td>Netropsin</td>
<td>20.04</td>
<td>02.40</td>
</tr>
<tr>
<td>Cu-Phen</td>
<td>20.38</td>
<td>34.96</td>
</tr>
<tr>
<td>Cu-SAHA-Phen</td>
<td>83.10</td>
<td>78.10</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ</td>
<td>14.60</td>
<td>13.40</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>15.30</td>
<td>14.20</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>SAHA</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>

$^aQ^a$ - concentration required to effect 50% removal of the initial fluorescence of bound dye.

4.3.3 Base-specific nucleic acid binding

In order to gain insight into base-specific nucleotide binding, thermal melting analysis and competitive EtBr displacement assays were performed on two synthetic alternating co-polymers of adenine-thymine, poly[d(A-T)$_2$] and guanine-cytosine, poly[d(G-C)$_2$]. Thermal melting ($T_M$) marks the midpoint in the melting process when equilibrium exists between the helical and single-stranded state. $T_M$ gives an insight into the strength of interaction between a drug and nucleic acid strand, as the stronger the drug-DNA interaction, the more energy required to denature the stabilized DNA relative to the untreated control ($\Delta T_M$). Actinomycin D substantially stabilised the thermal denaturation of poly[d(G-C)$_2$], netropsin stabilised the thermal denaturation of poly[d(A-T)$_2$] with both compounds exhibiting almost equal amounts of stabilisation (~12.60 °C) within these respective polymers. $T_M$ analysis of both agents highlights binding specificity with negligible stabilisation effects observed for the disfavoured polynucleotide. These findings are in agreement with previous studies in which Actinomycin D exhibits intercalative preference towards G-C rich copolymers$^{[4a]}$ and netropsin forms preferable interactions with a compressed minor groove in T-tracts of A-T rich DNA.$^{[6]}$ All of the Cu(II) chemotypes functioned to stabilize the thermal melting temperature of
poly[d(G-C)] to varying extents (~2 - 12.6 °C). Cu-SAHA-DPQ had the strongest stabilisation effect ($\Delta T_M$ 12.57 ± 0.73), Cu-SAHA-DPPZ following closely behind ($\Delta T_M$ 9.23 ± 1.56). Cu-Phen enhanced the thermal temperature of poly[d(G-C)] ($\Delta T_M$ 6.64 ± 1.58) thereafter followed by Cu-SAHA-Phen ($\Delta T_M$ 4.30 ± 0.51). Compared to poly[d(G-C)] all of the complexes had negligible or negative effects on thermal stabilization. The Cu(II) complexes bind both G·C and A·T co-polymers with $K_{app}$ values similar for both nucleotides tested.

**Table 15:** Effect of Actinomycin D, Netropsin, SAHA and Cu$^{2+}$ complexes on the thermal denaturation of poly[d(G-C)] and poly[d(A-T)]

<table>
<thead>
<tr>
<th>Complex</th>
<th>$C_{50}$ (µM)</th>
<th>$K_{app}$ (M(bp$^{-1}$))</th>
<th>$\Delta T_M$ ($^\circ$C)</th>
<th>$C_{50}$ (µM)</th>
<th>$K_{app}$ (M(bp$^{-1}$))</th>
<th>$\Delta T_M$ ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Netropsin</td>
<td>2.08</td>
<td>5.75 x 10$^7$</td>
<td>12.61 ± 0.61</td>
<td>&gt;500</td>
<td>NC</td>
<td>2.51 ± 0.51</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>&gt;500</td>
<td>NC</td>
<td>-0.31 ± 0.48</td>
<td>2.28</td>
<td>5.25 x 10$^7$</td>
<td>12.10 ± 0.95</td>
</tr>
<tr>
<td>Cu-Phen</td>
<td>17.25</td>
<td>6.94 x 10$^5$</td>
<td>-0.02 ± 0.29</td>
<td>7.09</td>
<td>1.69 x 10$^6$</td>
<td>6.64 ± 1.58</td>
</tr>
<tr>
<td>Cu-SAHA-Phen</td>
<td>121.40</td>
<td>9.86 x 10$^5$</td>
<td>-1.98 ± 0.15</td>
<td>115.80</td>
<td>1.03 x 10$^6$</td>
<td>4.30 ± 0.51</td>
</tr>
<tr>
<td>Cu-SAHA-DPQ</td>
<td>23.30</td>
<td>5.13 x 10$^6$</td>
<td>-1.07 ± 0.14</td>
<td>16.60</td>
<td>6.80 x 10$^6$</td>
<td>12.57 ± 0.73</td>
</tr>
<tr>
<td>Cu-SAHA-DPPZ</td>
<td>13.20</td>
<td>9.10 x 10$^5$</td>
<td>-1.03 ± 0.48</td>
<td>16.40</td>
<td>7.32 x 10$^5$</td>
<td>9.23 ± 1.56</td>
</tr>
<tr>
<td>Cu-SAHA-Phendio</td>
<td>&gt;150</td>
<td>NC</td>
<td>-0.39 ± 0.71</td>
<td>&gt;150</td>
<td>NC</td>
<td>-2.52 ± 0.66</td>
</tr>
<tr>
<td>Cu-SAHA-Bipy</td>
<td>&gt;150</td>
<td>NC</td>
<td>-1.72 ± 0.73</td>
<td>&gt;150</td>
<td>NC</td>
<td>-3.85 ± 0.51</td>
</tr>
<tr>
<td>SAHA</td>
<td>&gt;150</td>
<td>NC</td>
<td>-0.73 ± 0.48</td>
<td>&gt;150</td>
<td>NC</td>
<td>2.51 ± 0.51</td>
</tr>
</tbody>
</table>

$^aC_{50}$ - Concentration required to reduce fluorescence by 50%. $^bK_{app}$ - apparent binding constant ($K_{app} = K_c x 12.6/C_{50}$, where $K_c = 9.5 x 10^6$ M(bp$^{-1}$)). $^\circ T_M$ – difference in thermal melting ($\Delta T_M$) of drug treated nucleotide at $r = 0.1$ compared with drug-untreated nucleotide. NC – Not Calculated.

### 4.3.4 Chemical nuclease activity

The chemical nuclease activity of the complex series in the presence or absence of ROS specific scavengers was investigated to identify both (i) single and double stranded DNA damage and (ii) any possible ROS species involved giving rise to this DNA damage. Electrophoretic mobility shift assays
of the test complexes were performed with superhelical (SC, FI) pUC19 DNA. Concentrations of the test complexes were optimised in which a degradation profile could be visualised and representative DNA bands of single strand damage - open circular (OC, FII) and double strand damage - linear (LC, FIII) could be identified. Treatment of supercoiled FI pUC19 with Cu-Phen (Figure 27 lanes 1-4) results in FII and FIII bands at 1 µM & 2.5 µM respectively with complete degradation of the DNA strand occurring at 5 µM (data not shown). In order to identify the predominant ROS species involved in strand scission ROS-specific scavengers such as KI, NaN₃, Tiron and DMSO were added to the reaction mixture during the chemical nuclease experiments. Incubation of Cu-Phen with tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt or TH₂) (Figure 27, Lanes 13-16) a superoxide (O₂⁻⁻) scavenger,[⁷] significantly hinders the cleavage of FI to FII & FIII at 1 & 2.5 µM respectively. Similarly, DMSO, a hydroxyl radical (·OH) scavenger[⁸] had nearly an identical effect (Figure 27, Lanes 17-20) to tiron where cleavage activity from FI to FIII was also hindered at the same concentration. Incubation with KI[⁹] (H₂O₂ scavenger, Figure 27 Lanes 5-8) and NaN₃ (singlet oxygen (¹O₂) scavenger[¹⁰], Figure 27 Lanes 9-12) had marginal effects on the cleavage activity. This suggests two predominant ROS species formed during the reaction, mainly O₂⁻⁻ and ·OH.

![Figure 27 DNA cleavage reactions in the presence or absence of ROS specific scavengers: 400 ng of SC pUC19 was incubated in 80 mM HEPES for 30 min at 37°C with concentrations of 0.25, 0.5, 1 & 2.5 µM Cu-Phen in the presence of 25 mM NaCl, 0.5 mM Na-L-Ascorbate, 80 mM HEPES.](image-url)
The Cu-SAHA-Phen, Cu-SAHA-DPQ and Cu-SAHA-Phendio complexes possess similar nuclease activity to Sigman’s reagent, Cu-Phen\textsuperscript{[11]} (Figure 28) where complete degradation of FI to FII & FIII was observed at 2.5 µM for all three complexes. Similarly, the main ROS species involved in DNA damage were both O₂\textsuperscript{•−} and \( ^{\cdot}\)OH where both tiron and DMSO significantly inhibited the cleavage efficiency of FI to FII & FIII at 2.5 µM (Lanes 13-20), DMSO was the only scavenger which exhibited an inhibition of chemical nuclease activity of Cu-SAHA-Phendio possibly indicating \( ^{\cdot}\)OH is the only species involved in DNA damage for this complex. Unsurprisingly, KI and NaN\(_3\) had only marginal effects on chemical nuclease activity. Cu-SAHA-DPPZ was the next most active complex in the series with complete degradation of FI to FII and FIII observed at 10 µM, while O₂\textsuperscript{•−} was found to be a major ROS species, H\(_2\)O\(_2\) was also found to have a significant effect on the cleavage activity as KI inhibited the degradation of FI to FII and FIII at 10 µM – possibly due to the formation of a new Cu-oxo species. Cu-SAHA-Bipy was found to be a poor nuclease agent where at concentrations up to 15 µM, complete degradation of the DNA was not observed. Unsurprisingly, the SAHA ligand had no effect on chemical nuclease activity.
Figure 28 DNA cleavage reactions in the presence or absence of ROS specific scavengers: 400 ng of SC pUC19 was incubated in for 30 minutes at 37 °C with concentrations of 1, 2.5, 3.75 & 5 µM Cu-SAHA-Phen / Cu-SAHA-DPQ or Cu-SAHA-Phendiol; 2.5, 5, 10 & 15 µM Cu-SAHA-Bipy or SAHA; 1, 2.5, 5 & 10 µM Cu-SAHA-DPPZ; in the presence of 25 mM NaCl, 0.5 mM Na-L-ascorbate, 80 mM HEPES. Lanes 1 – 4: DNA + Complex; Lanes 5 – 8: + 10mM KI; Lanes 9 – 12: + 10mM NaN₃; Lanes 13 - 16: + 10 mM Tiron; Lanes 17 – 20: + 10% DMSO.

Figure 29 Radical scavenger interactions: Panel A: ROS scavengers utilized within this study. Panel B: Molecular structure of Tiron (TH₂). Panel C: Reaction equations of radical species and their respective scavengers.
4.4 Conclusion

We have successfully shown that incorporation of designer phenazine ligands DPQ and DPPZ into the Cu(II) complexes Cu-SAHA-DPQ and Cu-SAHA-DPPZ, greatly enhances their DNA binding properties towards ctDNA – both complexes possessing significant binding constants. Interestingly, the measured binding constants for both the Cu-SAHA-DPQ and Cu-SAHA-DPPZ complexes compare favourably with high-affinity Cu(II) binding constants previously reported in literature\(^{[12]}\) as well as the established minor groove binder Netropsin.

The Cu(II) chemotypes have a distinctive DNA binding mode. Both Cu-SAHA-DPQ and Cu-SAHA-DPPZ were found to quench both Hoechst 33258 and EtBr bound DNA to a similar level, which are significantly different to classic intercalating and minor groove binding agents. Thermal melting analysis revealed that these complexes preferentially stabilize the thermal denaturation of poly[d(G-C)\(_2\)] and also have a negligible effect on the thermal denaturation of 100% AT, in line with the intercalator Actinomycin D. Overall, it would appear that the Cu(II) chemotypes particularly Cu-SAHA-Phen, Cu-SAHA-DPQ and Cu-SAHA-DPPZ bind at both the major and minor groove, with these interactions enhanced with the presence of phenazine ligands DPQ and DPPZ.

Potent oxidative DNA cleavage activity was observed for the Cu-SAHA-DPQ, Cu-SAHA-Phen and Cu-SAHA-Phendio complexes, with similar activity observed relative to Cu-Phen. Scavengers were also employed to identify the main ROS species produced during the reaction of the Cu(II) chemotypes with DNA. O\(_2^{'-}\) and OH were found to be the predominant species with H\(_2\)O\(_2\) to a lesser extent, playing a role.
All of the above results suggest that these novel chemotypes, with potent in vitro cytotoxicities, HDAC inhibitory properties and DNA binding and chemical nuclease activities warrant further investigation with a view to generating a new class of therapeutic beyond those currently in use.

4.5 Chapter 4 References


Chapter 5 - Conclusions and Future Work
5.1 Conclusions

Through rational drug design, we have successfully synthesised and optimised, through a structure-activity relationship study, a series of Cu(II) complexes incorporating the HDACi SAHA and various designer DNA intercalating ligands.

Cu-Phen has been shown to be rather ‘promiscuous’ in that it induces cytotoxic properties across a broad range of cancerous, fungal and microbial cell lines.\textsuperscript{[1]} We have found that our complexes have promising \textit{in vitro} cytotoxic activity, some in the nM range, across a small panel of cancerous cell lines; of note is the fact that they appear to have selective cytotoxicity towards cell lines possessing mutant \textit{p53} genes and selective activity towards certain bacterial and fungal strains. These complexes, compared to the free ligands alone and Cu-Phen, may therefore differ in their mechanism of action and may thus possibly overcome some of the drawbacks associated with existing therapies.

We have also shown that the Cu(II) complexes are capable of inhibiting HDAC function, comparable if not better than the clinically used HDACi SAHA within the same timeframe at isotoxic concentrations, under cellular conditions. It has been well documented in the literature that hydroxamates are subject to \textit{in vivo} metabolic degradation resulting in shorter half-lives;\textsuperscript{[2]} a limitation often associated with the clinical development of hydroxamate-based drugs. As the hydroxamate functionality is directly coordinated to the Cu(II) in the Cu chemotypes described, the Cu(II) ion may well serve to protect the HDACi hydroxamate functionality until such time as the chemotype reaches its biological target whereupon the HDACi is released following reduction from Cu(II) to Cu(I), ultimately potentially improving the pharmacokinetic profile of hydroxamate-based HDAC inhibitors. An added advantage therefore of the complexes described is that coordinating HDACi with metal cations such as Cu(II) may well widen their therapeutic utility.
While Cu-Phen has been shown to intercalate DNA base pairs within the minor groove of A-T rich regions of DNA, our complexes have been found to bind DNA with high binding constants whilst also demonstrating selectivity in stabilizing G-C rich regions of DNA. Our complexes have also been shown to induce oxidative DNA damage through the generation of ROS species, mainly O$_2^{-}$ and `OH and H$_2$O$_2$, to a lesser extent, also playing a role.

In conclusion, we have been successful in our attempts to develop an innovative class of multi-functional Cu drug candidates possessing potent HDAC inhibitory activities, low microM and even nanoM cytotoxicities, DNA binding and chemical nuclease properties. They also possess anti-bacterial and anti-fungal properties. These chemotypes warrant further studies to more fully understand their mechanism of action with a view to ultimately generating a new class of metallochemotherapeutic beyond those currently in use.

5.2 Future work

Given the potential of the chemotypes described, further biological studies are warranted to better understand their mechanism of action. For example, in chapter 4, we described the chemical nuclease activity of the complex series. These experiments however were performed on plasmid pUC19 DNA. In order to assess the capability of the complex series to induce oxidative stress under cellular conditions, we propose that an additional series of cellular experiments be performed to measure oxidative stress induced by these complexes in SK-OV-3 cells.

We have conducted some preliminary investigations into induction of oxidative stress - via measurements of incorporation of a thymidine analogue, 5-ethynyl-2'-deoxyuridine (EdU), (Figure 30 - Panel B) into SK-OV-
3 cells. Under normal cellular conditions, a sample containing EdU is added to the cell medium which is then uptaken by cells and incorporated into the DNA strand during active DNA synthesis. EdU, once incorporated into the DNA strand, can then be conjugated to a fluorescent dye molecule at the alkyne moiety of EdU via a Cu(I)-catalysed azide alkyne Huisgen cycloaddition “click” reaction. The fluorescent intensity of the conjugated dye molecule can then be measured and quantified via flow cytometric measurements. Treatment of SK-OV-3 cells with Cu-SAHA-DPQ and Cu-SAHA-DPPZ at IC_{25} and IC_{50} values respectively, followed by EdU treatment, allowed us to investigate the impact of our complexes on DNA synthesis. Preliminary results indicated lower EdU incorporation (~50% reduction) as compared to control (untreated) cells (Figure 30 Panel A). This suggests that the Cu complexes affect DNA synthesis although further studies would be required to substantiate this finding. One such study might include a comparison between EdU incorporation with known oxidative DNA stress markers such as 8-Oxo-dG.\[3\]

**Figure 30 Cu(II) complexes inhibit DNA Synthesis:** Panel A: SK-OV-3 cells were seeded at an initial density of 6 × 10^4 cells ml^{-1} overnight. Cu(II) complexes were added to SK-OV-3 cells at isotoxic concentrations corresponding to the IC_{25} or IC_{50} value and incubated for 24 hours. Inhibition of DNA synthesis by the Cu(II) complexes was measured via 5-ethynyl-2'-deoxyuridine (EdU – Panel B). EdU incorporation was measured according to the protocol recommended by the manufacturer of the EdU cell proliferation kit (Baseclick GmBH). Analysis was performed on a Guava EasyCyte HT flow cytometer. Datapoints representative of average of triplicate measurements, ± SD; N=2.
5.3 Derivative syntheses

The Cu chemotypes described possess enhanced cytotoxicity as compared to Cu-Phen or SAHA alone; the presence of the metal ion as well as the incorporation of designer DNA intercalating ligands clearly playing a role in enhancing the cytotoxicity profile of these complexes. Whilst SAHA was the first hydroxamate-based inhibitor to enter the clinic as a treatment for lymphoma, Belinostat (US trade name Beleodaq®), another hydroxamate-based HDACi inhibitor more recently entered the clinic receiving USA FDA approval in July 2014. We have demonstrated that by changing the DNA intercalating ligand in the Cu chemotypes described, we can alter the biological properties of the resulting complexes with Cu-SAHA-DPQ and Cu-SAHA-DPPZ showing optimum activities. Further work should include developing Cu-X-DPQ and DPPZ candidates (keeping the intercalating ligand constant) while changing the HDACi (X) where X is either Belinostat or other promising HDACi currently undergoing clinical trials, with a view to further expanding and optimising this new class of derivatives.

Figure 31 Functionalised Cu(II) drug candidates: Chemical structures of proposed complexes for further derivitisation.

We have already generated preliminary data in this regard. We have successfully synthesized and characterised the [Cu(Belinostat-1H)(Phen)]ClO₄
(Cu-Bel-Phen) complex using a similar procedure to that described for the Cu-SAHA-Phen reported in chapter 2.

The development of Cu-X-DPQ and Cu-X-DPPZ, where X is Belinostat and other hydroxamate-based HDACi, should therefore be relatively straightforward. We suggest that this new series be investigated and their biological activities evaluated for anti-cancer activity.

We also propose that once an optimum complex has been identified, that in vivo studies be conducted with a view to bringing these chemotypes to the next stage in the drug development process.
5.4 Chapter 5 references


Appendix
A.I  $^1$H NMR – Ligands

Figure 32: $^1$H NMR spectra of SAHA (7 – 10.4 ppm)
Figure 33: $^1$H NMR spectra of SAHA (1 - 2.7 ppm)
Figure 34: $^1$H NMR of Phendione (7.5 – 9.1 ppm)
Figure 35: $^1$H NMR of DPQ (7.4 – 9.9 ppm)
Figure 36: $^1$H NMR of DPPZ (7.6 – 10.1 ppm)
A.II IR spectra – Ligands

Figure 37: IR spectra of SAHA.
Figure 38: IR spectra of Phen.
Figure 39: IR spectra of Phendio.
Figure 40: IR spectra of Bipy.
Figure 41: IR spectra of DPQ.
Figure 42: IR spectra of DPPZ.
**Figure 43:** IR spectra of Cu-SAHA-Phen.
Figure 44: IR spectra of Cu-SAHA-Phendio.
Figure 45: IR spectra of Cu-SAHA-Bipy.
Figure 46: IR spectra of Cu-SAHA-DPQ.
Figure 47: IR spectra of Cu-SAHA-DPPZ.
A.IV Mass spectra – Complexes

Figure 48: Mass spectrum of Cu-SAHA-Phen.

Figure 49: Mass spectrum of Cu-SAHA-Bipy.
Figure 50: Mass spectrum of Cu-SAHA-DPO.

Figure 51: Mass spectrum of Cu-SAHA-DPPZ.
Figure 52: Mass spectrum of $[\text{Cu(Belinostat}^\text{+})(\text{Phen})]\text{ClO}_4$.
A.V Dose-Response curves (SK-OV-3 cells, Complexes & SAHA)

Figure 53: Dose response curves, complexes & SAHA (SK-OV-3) 24-72 Hour timepoints.
A.VI  Dose-Response curves (DU145 cells, Complexes & SAHA)

Figure 54: Dose-Response curves, complexes & SAHA (DU145) 24 & 72 Hrs.
Figure 55: Dose-Response curves, complexes & SAHA (DU145) 24 & 72 Hrs.
A.VII Dose-Response curves (Doxorubicin, SK-OV-3; DU145 & MCF-7)

Figure 56: Dose-Response curves, Doxorubicin (SK-OV-3, DU145 & MCF-7) 24 - 72 Hrs.