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Advances in Cobalt Complexes as Anticancer Agents

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Abstract

The evolution of resistance to traditional platinum-based anticancer drugs has compelled researchers to investigate the cytostatic properties of alternative transition metal-based compounds. The anticancer potential of cobalt complexes has been extensively studied over the last three decades, and much time has been devoted to understanding their mechanisms of action. This perspective catalogues the development of antiproliferative cobalt complexes, and provides an in depth analysis of their mode of action. Early studies on simple cobalt coordination complexes, Schiff base complexes, and cobalt-carbonyl clusters will be documented. The physiologically relevant redox properties of cobalt will be highlighted and the role this plays in the preparation of hypoxia selective prodrugs and imaging agents will be discussed. The use of cobalt-containing cobalamin as a cancer specific delivery agent for cytotoxins will also be described. The work summarised in this perspective shows that the biochemical and biophysical properties of cobalt-containing compounds can be fine-tuned to produce new generations of anticancer agents with clinically relevant efficacies.

Introduction

The fortuitous discovery of cisplatin's anticancer activity spawned what is today the field of metal-based chemotherapeutics.¹ Platinum-based drugs, cisplatin, carboplatin and oxaliplatin are routinely used alone or in combination with other agents, to treat a variety of malignancies such as testicular, ovarian, lung, colorectal, head and neck cancer.²⁻⁷ The platinum agents induce their therapeutic effect by entering cancer cells and binding to DNA, in a covalent, bifunctional manner.⁸ This event distorts DNA secondary structure and blocks DNA transcription and replication, which through a complex network of chemical signals triggers apoptosis.⁹⁻¹¹ Despite the widespread clinical use of platinum-containing drugs, their use is limited, due to acquired or inherent resistance, toxicity side-effects arising from non-specificity, and their inability to prevent cancer relapse.¹²⁻¹⁴ These drawbacks have fuelled research into the development of non-platinum containing metallopharmaceuticals.¹⁵ Several ruthenium-, gold-, gallium-, titanium-, and arsenic-based compounds have been investigated for their anticancer potential.¹⁶⁻²¹ Although none of these compounds have been approved clinically, significant advancements have made in their development including clinical studies with some of the most promising candidates. There are several reviews that highlight the successes and failures of non-platinum anticancer agents, however, to the best of our knowledge there is no report dedicated solely to the anticancer activity of cobalt complexes of all oxidation states. It should be noted that reviews devoted to the general use of cobalt complexes in medicine have been published.^{22, 23} This perspective aims to provide a timely account on the anticancer properties of reported cobalt compounds, with a particular emphasis on their proposed mechanism(s) of action.

Birth of biologically active cobalt complexes

Cobalt is an essential trace element found in all animals. Cobalt plays a crucial role in several biologically important processes, and is predominately found in the form of vitamin B12 (cobalamin).²⁴ Within

cobalamin, cobalt exists in the +1 oxidation state (although it can undergo oxidation to the +2 and +3 states) and adopts an octahedral geometry. The cobalt(I) ion is equatorially coordinated to four nitrogen atoms of a corrin ring, and axially to 5,6-dimethylbenzimidazole and either cyanide, hydroxide, methyl, or 5'-deoxyadenosyl groups.²⁵ The different forms of cobalamin are necessary for proper formation of red blood cells, DNA synthesis and regulation, and the maintenance of normal brain and nerve function.²⁴ There is also evidence implicating cobalamin in fatty acid and amino acid metabolism.²⁴ Given the prominent role of cobalt in biological processes, humans have evolved mechanisms to overcome cobalt overload.²⁶ Cobalt is thus less toxic to humans than non-essential metals like platinum. This fact prompted researchers to investigate cobalt-containing compounds as less toxic alternatives to platinum-based anticancer drugs. The first biological study conducted with cobalt complexes was carried by Dwyer *et al.* in 1952.²⁷ The toxicity of tris-acetylacetane cobalt(III) (1), racemic or optical isomers of tris-ethylenediamine cobalt(III) nitrate (2a-b), 1;8-bis(salicylideneamino)-3; 6-dithiaoctane cobalt(III) chloride (3), 1;10-bis(salicylideneamino)-4; 7-dithiadecane cobalt(III) iodide (4), and tris-glycine cobalt(III) (5) (Figure 1) in mice was determined after intraperitoneal administration. The dose required to induce death was reasonably high for all of the cobalt complexes tested (ranging between 75-165 mg/ kg), highlighting the low systemic toxicity of cobalt. This seminal study also found that 4 exhibited bacteriostatic and bacteriocidal activity against *E. coli* and *S. haemolyticus* in the micromolar range. Unlike cisplatin, where biological activity in *E. coli* led to antiproliferative studies in mammalian cells and subsequent anticancer drug development, the antitumour properties of 4 or its derivatives were not investigated further. Instead, the ability of cobalt complexes to induce death (in mice) by paralysis and respiratory failure²⁷ motivated Dwyer and co-workers to assess the ability of a series of cationic, anionic, and neutral cobalt complexes containing ammonia, nitrite, ethylenediamine, bipyridine, phenanthroline and terpyridine ligands, to block neuromuscular transmission at the diaphragm-phrenic nerve junction and the rectus abdominis muscle, in rats and toads respectively.²⁸ The cobalt complexes displayed varying levels of curariform activity depending on the overall charge, and were proposed to induce their effect by direct competition with acetylcholine at the junctional region(s). The cobalt phenanthroline complexes investigated in this study were later established as potent antibacterial agents.²⁹ While these early studies did not contribute directly to the development of cobalt anticancer agents, they helped researchers realise the vast biological potential of cobalt-containing compounds. Indeed, several cobalt compounds have now been prepared with therapeutically relevant antifungal, antibacterial, and antiprotozoal properties.³⁰⁻³⁴

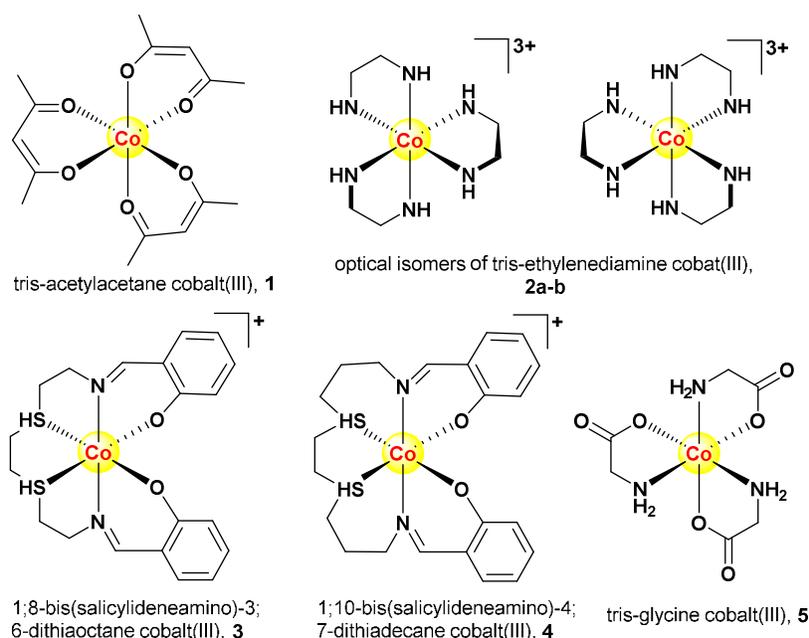
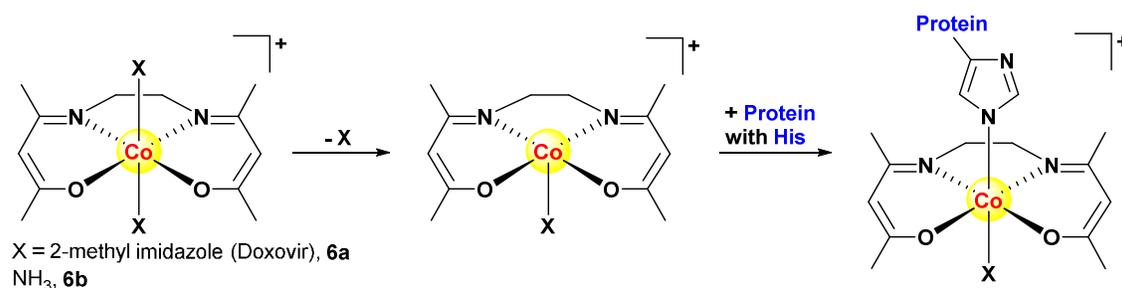


Figure 1. Chemical structures of cobalt(III) coordination complexes used in early biological studies.

Histidine targeting Co(III) Schiff base complexes and related compounds

The most significant medical advancement in terms of cobalt compounds thus far, is the clinical development of Doxovir (CTC-96) for Herpes labialis (or herpes simplex virus 1).³⁵ Doxovir (**6a**) is a cobalt(III) complex of bis(acetylacetonate)ethylenediimine (acacen), with two axially coordinated 2-methylimidazole ligands. This experimental drug exhibits potent microbicidal effects on drug resistant strains of the herpes virus, and has recently completed phase II clinical trials. The exact mechanism of action of Doxovir is unknown, but it is speculated to induce its therapeutic effect by covalently binding to histidine residues in the active site of a viral enzyme that is crucial for Herpes replication. Biophysical studies with a simpler, ammonia containing analogue (**6b**) revealed that histidine coordination most likely occurs via dissociative displacement of the axial ligands (**Scheme 1**).³⁶ This class of compounds was also shown to irreversibly inhibit thermolysin and thrombin activity by a similar mechanism.³⁷ Meade and co-worker showed that by attaching specific peptides and oligonucleotides to the Co(III)-acacen core, histidine residues in (zinc-finger) proteins could be targeted with greater precision. This strategy has been utilised to inhibit several transcription factors (such as those involved in Snail and Hedgehog signalling) associated with cancer progression.³⁸⁻⁴⁰ Preparation of novel Co(III) Schiff base bioconjugates targeted to other transcription factors involved in tumour progression and metastasis could prove to be therapeutically very appealing.



Scheme 1. The proposed mechanism of action of Co(III)-acacen complexes, including Doxovir. It is postulated that one of the axial ligands is lost in a dissociative manner, followed by covalent binding to histidine residues within the active site of key enzymes or signalling proteins.

Recently a number of simple cobalt(II) and cobalt(III) Schiff base complexes have been reported to display reasonable anticancer activity. The Schiff base complex of cobalt(II) containing a 4-(4-aminophenyl) morpholine derivative displayed very poor activity against hepatocellular carcinoma cells (HepG2), with an IC₅₀ value in the millimolar range.⁴¹ On the other hand, cobalt(II) complexes containing 2,6-bis(2,6-diethylphenyliminomethyl)pyridine exhibited better cytotoxicities against colorectal adenocarcinoma (HCT-15) and cervix adenocarcinoma (HeLa) cells, with IC₅₀ values ranging from 45-100 μM.⁴² A cobalt(III) complex containing the tridentate Schiff base ligand derived from the reaction of salicylaldehyde and ethylene diamine displayed moderate activity (IC₅₀ < 100 μM) against human breast cancer cells (MCF-7).⁴³ Cobalt(III) complexes bearing two [2-(2-hydroxybenzylideneamino) phenol] ligands inhibited HeLa cell growth, but to a lower extent than cisplatin.⁴⁴ In contrast, a water soluble, ligand-bridged cobalt(II) coordination polymer containing 2-oxo-1,2-dihydroquinoline-3-carbaldehyde (isonicotinic) hydrazine ligands displayed micromolar toxicities against a panel of cancer cells (HeLa, HEP-2, and A431), comparable to, and in some cases better than, that of cisplatin.⁴⁵ Remarkably this coordination polymer exhibited up to 100-fold lower toxicity against non-cancerous mouse embryonic fibroblast cells (NIH 3T3). Given the large discrepancies in biological activity of cobalt Schiff base complexes, systematic structure-activity relationship studies need to be carried out to determine the true pharmacological potential of this class of compounds. Other cobalt coordination complexes such as those containing bidentate ligands (like phenanthroline and bipyridine) also exhibit promising in vitro activities against cancer cells.^{46, 47} Notably,

those with surfactant-like properties have been shown to induce DNA damage and apoptosis in cancer cells at therapeutically attractive concentrations.⁴⁸⁻⁵⁰

Cobalt carbonyl-nonsteroidal anti-inflammatory drug (NSAIDs) conjugates

Dicobalt(0) hexacarbonyl, $[\text{Co}_2(\text{CO})_6]$ complexes with alkyne ligands are valuable reagents in organic chemistry. For instance, they are used in the Pauson-Khand reaction for the synthesis of α,β -cyclopentenones, and the Nicholas reaction for the stabilisation of propargylic cations, which are reacted with nucleophiles to generate alkylated alkynes. In terms of biological applications, dicobalt(0)hexacarbonyl-alkyne complexes were initially used as labelling agents.⁵¹ Ethinylestradiol was tethered to $[\text{Co}_2(\text{CO})_6]$ and used to monitor the binding of steroids to estrogen receptors in breast cancer cells. The formation of a α -cation in close proximity to the $[\text{Co}_2(\text{CO})_6]$ unit enabled the complex to bind covalently to nucleophilic centres within the substrate binding site of the estrogen receptors.⁵² The potential for using such cationic intermediates to target and damage DNA (in cancer cells), motivated Jung⁵³ and then Ott and Gust *et al.* to investigate the anticancer properties of $[\text{Co}_2(\text{CO})_6]$ -acetylene complexes.⁵⁴ Early structure-activity relationship studies found that the ability of the complexes to inhibit cancer cell proliferation was strongly dependent on the chemical nature of the acetylene moiety.^{55,56} Systematic *in vitro* cytotoxicity studies showed that Co-ASS (**7**), a $[\text{Co}_2(\text{CO})_6]$ complex with an alkyne-bearing aspirin derivative, was the most potent compound among those tested (**Figure 2**). Control experiments with non-functional derivatives, cobalt(II) chloride, and $[\text{Co}_2(\text{CO})_8]$ proved that **7** did not cause non-specific toxicity and that activity was due to the intact complex.⁵⁴ Cell uptake studies implicated that activity was largely due to the hydrophobicity endowed by the aspirin unit. Subsequent work with related compounds of differing lipophilicities, however, revealed that there was no correlation between hydrophobicity and toxicity.⁵⁶ Cell internalisation efficiency was therefore not considered to be a major determinant of **7** activity. Furthermore, analytical studies with salmon testes DNA and nuclei extracted from breast cancer cells (MCF-7 and MDA-MB-231) treated with **7**, found little cobalt content, thus ruling out DNA interactions as a mode of action.⁵⁶

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are known to exhibit their anticancer effect by inducing cell cycle arrest, apoptosis, and angiogenesis suppression, through the inhibition of cyclooxygenase (COX) enzymes.⁵⁷ Therefore the ability of **7** to perturb COX function was investigated. Relative to aspirin, **7** markedly enhanced COX-1 and COX-2 inhibition resulting in greater antiproliferative activity against breast adenocarcinoma cells (MDA-MB-231).⁵⁸ The disparity in pharmacological properties of aspirin and **7** has been postulated to result from their differing interactions with COX-2. While aspirin and its acetylene derivative induce acetylation of serine residues within the active site, **7** promotes acetylation of lysine side chains within the entrance channel leading to the active site, causing reduced substrate entry.⁵⁸ Another proposed mode of COX inhibition involves CO poisoning of the heme group.⁵⁴ In an attempt to amplify COX inhibition through this mechanism, aspirin derivatives with higher cobalt and CO content were prepared.⁵⁹ The cytotoxic activity of the $[\text{Co}_4(\text{CO})_{10}]$ -containing aspirin conjugate, Prop-ASS- Co_4 (**8**) (**Figure 2**), was marginally enhanced compared to **7** in colorectal adenocarcinoma cells (HT-29) and retained in breast cancer cells (MCF-7 and MDA-MB 231). Unfortunately, elongation of the alkyne functionality (But-ASS- Co_4 , **9**), and incorporation of an additional aspirin unit (Di-ASS- Co_4 , **10**), did not improve biological activity (**Figure 2**). These results suggested that COX-interference might be of lesser importance in terms of the mode of action of aspirin dicobalt-carbonyl complexes than first thought. Indeed, **7** also activates pathways independent or downstream of COX such as caspase-3 cleavage and matrix metalloproteinase dysfunction.⁵⁸ The data available at present indicates that the mechanism of action of **7** (and related compounds) is multifactorial. Despite not knowing the exact mode of action of **7** and its derivatives, the compounds have been used in combination with other cytostatics agents to tackle tumours. When combined with imatinib, a tyrosine kinase inhibitor, **7** exhibited an additive or synergistic effect on the proliferative inhibition of acute and chronic myeloid leukemia cells (HL-60, LAMA-84, and CML-T1).⁶⁰

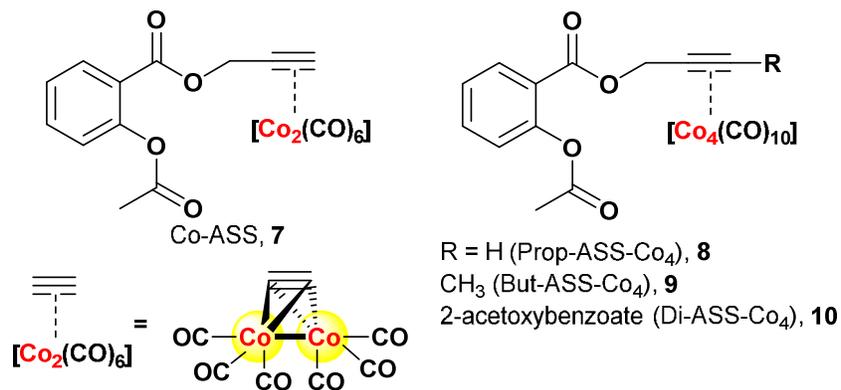


Figure 2. Chemical structures of biologically active cobalt carbonyl clusters attached to alkyne-bearing aspirin derivatives. The most potent complex in this series, Co-ASS, **7** blocks cancer cell proliferation in a multifactorial manner (which includes COX-2 inhibition).

Given the impressive biological activity of **7** and other dicobalt-carbonyl complexes, several small molecules and biomolecules containing dicobalt-carbonyl moieties have been prepared and investigated for their anticancer activity (**Figure 3**). Overall, the results of these studies have proved to be somewhat disappointing. Fructopyranose derivatives coupled to $[Co_2(CO)_6]$ through an alkyne functionality (**11a-c**), reminiscent of auranofin (a gold-based NSAID) displayed modest activity ($IC_{50} > 20 \mu M$) against human breast cancer cells (MCF-7).⁶¹ The attachment of dicobalt octacarbonyl, $[Co_2(CO)_8]$ to deoxyuridines analogues (**12a-h**) afforded compounds with slightly better antiproliferative activity (IC_{50} values ranging from 5–50 μM) against human breast cancer cells (MCF-7 and MDA-MB-231).⁶² The incorporation of $[Co_2(CO)_6]$ into a ferrocenyl-chromone complex also yielded a weak cytostatic agent (**13**).⁶³ In contrast, the integration of $[Co_2(CO)_6]$ into the scaffold of a targeting peptide, namely leucine-enkephalin (primary amino acid sequence Tyr-Gly-Gly-Phe-Leu) (**14**), produced the first organometallic peptide bioconjugate to show significant toxicity against tumour cells (cervix adenocarcinoma, HeLa and hepatocellular carcinoma, HepG2).⁶⁴ This discovery could pave the way for the bioconjugation of cytotoxic metal fragments to other targeting peptides, in an effort to improve their uptake into cancer cells.

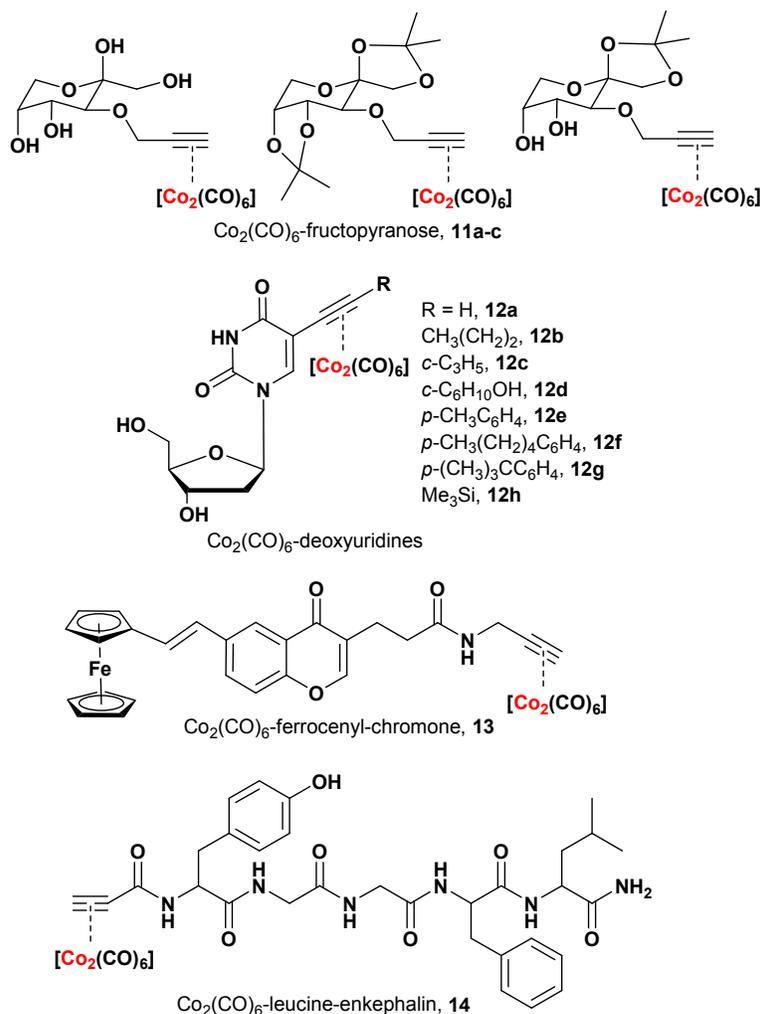


Figure 3. Chemical structures of biologically relevant molecules containing a $[\text{Co}_2(\text{CO})_6]$ unit which have undergone cell-based studies. The metal-peptide bioconjugate, **14** was the first such complex to show significant antiproliferative activity against cancer cells.

More recently, a number of cobalt(II) complexes attached to NSAIDs, such as mefenamic acid (**15a-c**), naproxen (**16a-c**), and tolfenamic acid (**17a-c**) have been prepared, and their interactions with important biomolecules have been probed (**Figure 4**).⁶⁵⁻⁶⁷ In terms of structure, the complexes consist of a Co(II) ion bound to the corresponding deprotonated NSAID via the carboxylato oxygen, and one or two polypyridyl ligand(s) (pyridine, 2,2'-bipyridine or 1,10-phenanthroline). The complexes displayed strong affinity for duplex DNA and HSA, and high antioxidant activity (high scavenging efficacy against hydroxyl and superoxide radicals). Unfortunately, the anticancer potential and in vitro COX inhibitory effect for this promising class of compounds cannot be gauged as cell-based studies are yet to be reported.

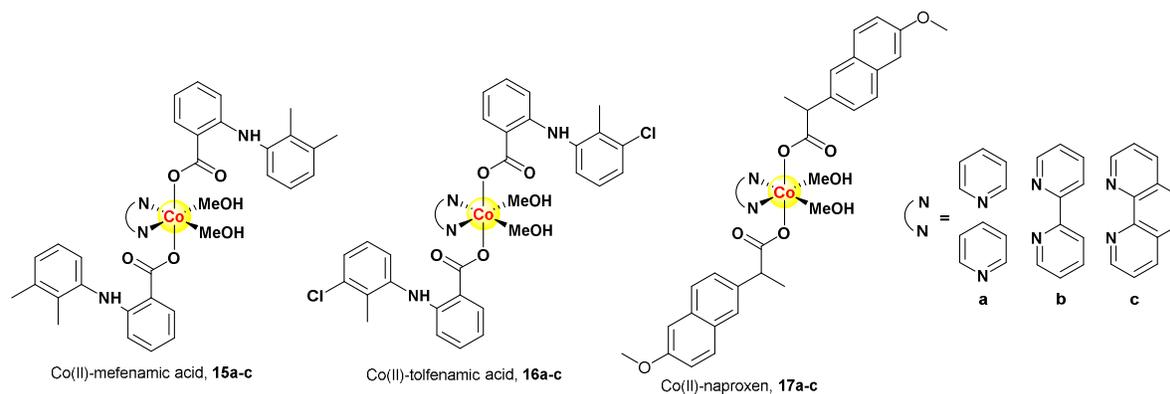
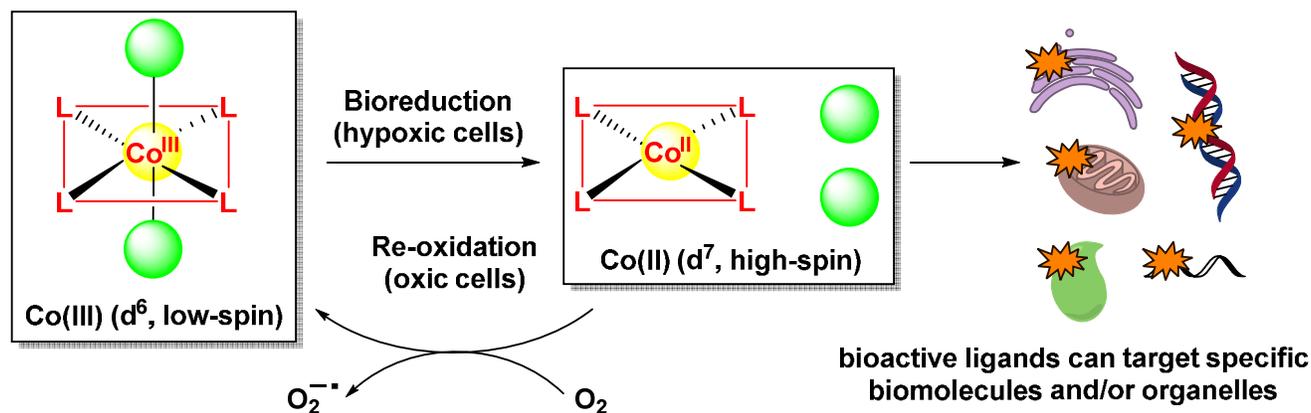


Figure 4. General chemical structure of cobalt(II)-NSAIDs complexes with polypyridyl ligands. The complexes exhibit strong interactions with DNA and proteins in cell-free systems. The in vitro potential of these complex are yet to be fully explored.

Hypoxia activated cobalt(III) prodrugs

Pro-drugs are inert agents that can be activated by external stimuli such as light, ionising radiation, or reducing environments.⁶⁸ As hypoxic cancer cells are resistant to conventional radiotherapy and chemotherapy, hypoxia-activated prodrugs are highly sought-after.^{69, 70} The difference in reactivity of the accessible oxidation states of cobalt (II and III) has enabled the development of cobalt(III) prodrugs that can undergo bioreductive activation in hypoxic regions (within solid tumours).²² Bioreductive activation is a process whereby an inert agent undergoes reduction upon cell entry, releasing one or more bioactive agents (**Scheme 2**).⁷¹ Cobalt(III) complexes usually adopt octahedral geometries and are relatively inert, owing to the high crystal field energy stabilisation of Co(III) (d^6 , low-spin). Co(II) complexes (d^7 , high-spin) on the other hand are more labile and susceptible to ligand substitution. The intracellular reduction of cobalt(III) prodrugs is thought to be mediated by endogenous intracellular reductases such as xanthine oxidase, NADPH, or cytochrome P450.^{72, 73} Bioreductive activation can only occur if the reduction potential of the initial Co(III) complex is compatible with the cytosolic reduction potential in cells (-200 mV to -400 mV).⁷⁴ A major concern for hypoxia-activated prodrugs is selective activation in cancer cells. Traditional hypoxia-activated prodrugs (based on nitroimidazoles, quinones, or aromatic N-oxides) rely on re-oxidation of the active form to the inert form in normal cells but not in cancer cells.⁷⁵ Healthy tissue, however, can also be highly hypoxic under certain condition, thus to completely avoid toxic side effects it is essential for prodrugs to have high activation thresholds.⁷⁶ As the reduction potential of cobalt(III) complexes can, in theory, be fine-tuned by ligand alterations to coincide with the cytoplasmic environment in cancer cells (rather than normal cells), such agents offer a viable route to develop truly selective prodrugs. Over the last three decades, several cobalt(III) prodrug candidates with reduction potentials ranging from 0 mV to -1400 mV (vs. NHE), have been reported. Here we will discuss the most extraordinary examples.



Scheme 2. The proposed mechanism of action of Co(III) prodrugs. Upon internalisation by hypoxic cells, cobalt(III) complexes are thought to undergo bioreductive activation, whereby the Co(III) centre is reduced to Co(II), and one or more bioactive ligand(s) are released. In oxic cells, the active Co(II) form is hypothesised to undergo re-oxidation back to the inert Co(III) form.

Cobalt(III)-mustard agents

Teicher *et al.* discerned that Co(III) complexes containing amine and nitro ligands were very effective radiation sensitizers for hypoxic mammary carcinoma cells (EMT6).⁷⁷ Further research into Co(III)-nitro complexes, found that those containing acetylacetonate (acac) and nitrogen mustard ligands, such as bis(2-chloroethyl)amine (BCA) (**18**), were particularly effective against cultured murine tumour cells (**Figure 5**).⁷⁸ Nitrogen mustard agents are a class of clinically approved anticancer drugs,⁷⁹ that induce their therapeutic effect by alkylating purine DNA bases.⁸⁰ In the oxidised state, **18** is inert, as the nitrogen lone pair belonging to the BCA component is involved in coordination to cobalt and thus unavailable for intramolecular formation of an aziridinium ion, which is a prerequisite for DNA alkylation. In the reduced state, BCA is released from the resulting Co(II) complex, enabling a cytotoxic effect. Cytotoxicity studies showed that **18** exhibited higher toxicity towards oxygenated cells than hypoxic cells, and strong radiosensitisation of hypoxic mammary carcinoma cells (EMT6).⁷⁸ Encouragingly, from a clinical point of view, **18** overcame mustard agent related resistance in squamous cell carcinoma (SCC-25/HN2) lines, produced a > 60-day increase in the life span of mice bearing leukemia cells (L1210), and delayed (by several days) tumour growth in mice bearing fibrosarcoma cells (FSa1C).⁷⁸

Ware and co-workers used the same rationale design employed for **18** to develop Co(III)-nitro complexes with alkylating aziridine groups, with the hope of selectively releasing the bioactive ligands under hypoxic conditions.⁸¹ Unfortunately, cell-based studies revealed that the complexes underwent facile reduction, leading to uncontrolled release of the aziridine groups. Bidentate mustard ligands, *N,N'*-bis(2-chloroethyl)ethylenediamine (BCE) and *N,N'*-bis(2-chloroethyl)ethylenediamine (DCE), were utilised to prepare a series of kinetically stable Co(III)-acetylacetonate complexes (**19a-c** and **20a-c**) capable of selectively killing hypoxic cells (**Figure 5**).⁸²⁻⁸⁴ The cytotoxicity trend of the Co(III)-BCE and Co(III)-DCE complexes matched that of the free mustard agents, suggesting that toxicity was due to the release of the mustard agents. Further cytotoxicity studies with stirred suspensions found that the most effective complex, **19b**, was 6-fold more cytotoxic towards Chinese hamster ovary fibroblast cells (AA8 and UV4) grown in hypoxic conditions than the same cells cultured in aerobic conditions. More recently, the hypoxic selectivity of **19b** was reported to be as high as 20.3-fold.⁸⁵ This complex also displayed high activity against mammary carcinoma cells (EMT6) in intact spheroids. Spheroids have hypoxic centres, therefore **19b**-induced toxicity is believed to result from DCE release within the core followed by back-diffusion to the outer regions.⁸² Structure-activity relationship studies found that the nature of the ancillary ligand,

acetylacetonate, was crucial to hypoxic selectivity. While Co(III)-DCE complexes with unsubstituted acetylacetonate displayed no hypoxic selectivity, methyl and ethyl analogues showed substantial selectivity (up to one order of magnitude).⁸²

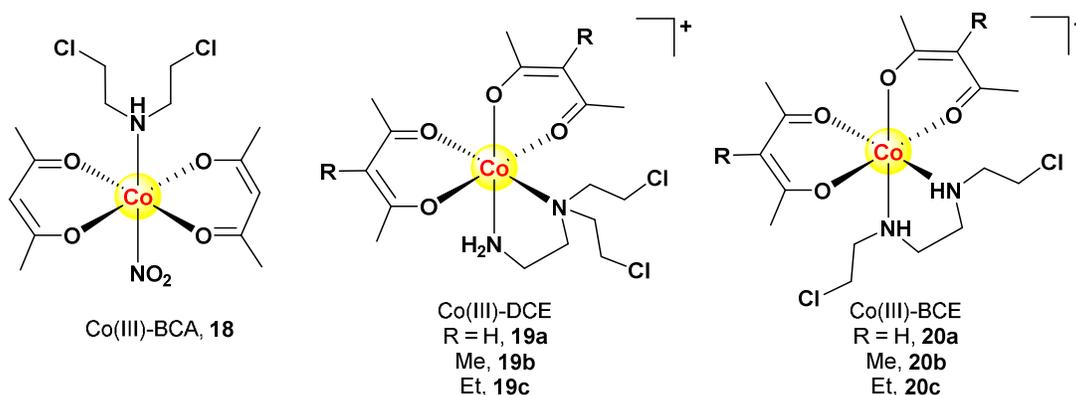


Figure 5. Chemical structures of hypoxia selective Co(III) prodrugs with acetylacetonate and mono- or bidentate nitrogen mustard ligands. Complex **19b** is the more hypoxic selective complex within this series.

Replacement of acetylacetonate with bis-tropolonate yielded complexes (**21**) that suffered from facile cellular reduction under oxic and hypoxic conditions, owing to their high reduction potentials (**Figure 6**).⁸⁶ For this reason, unsubstituted bis-tropolonate was deemed to be an unsuitable ancillary ligand for generating hypoxic selective Co(III) complexes. However, the introduction of electron withdrawing groups into the tropolone ring could generate hypoxia-selective complexes. Replacement of acetylacetonate with anionic carbonato and oxalato ligands afforded Co(III)-DCE complexes (**22** and **23**) with reasonable hypoxic selectivity (2-4-fold against UV4 cells) (**Figure 6**).⁸⁷ Nevertheless, the hypoxic selectivity of these complexes was still inferior to that of **19b** under the same conditions.

In order to understand the mechanism of hypoxic selectivity of the Co(III)-DCE series (**19a-c**), detailed pulse radiolysis studies were conducted with the lead complex, **19b**.⁸⁸ This work suggested that hypoxic selectivity did not arise from redox cycling i.e. the re-oxidation of the active form (Co(II) species) by oxygen in oxic cells but not in hypoxic cells. Instead this study showed that following reduction of the Co(III) centre, the alkylating ligand was released independent of oxygen status. Therefore hypoxic selectivity was proposed to result from competition between the activated Co(II) species and oxygen for intracellular reductants. The fact that redox cycling was not a major determinant of hypoxic selectivity, suggested that the stability of the initial Co(III) complex may be a controlling factor. In vivo studies (in mice) showed that **19b** was metabolically unstable, and thus displayed high systemic toxicity and low bioreductive radiosensitisation. In an attempt to develop physiologically stable Co(III)-prodrugs with enhanced hypoxic selective, tridentate mustard agents were used (**Figure 6**).⁸⁵ However Co(III)-acetylacetonate complexes containing *N,N*-bis(2-chloroethyl)diethylenetriamine (DCD) (**24**) only exhibited modest hypoxic selectivity (5-fold) in clonogenic studies. Moreover, larger amounts **24** (up to 300-fold more) were required to induce the same inhibitory effect on AA8 and UV4 cell growth as Co(III) analogues containing bidentate mustard ligands.

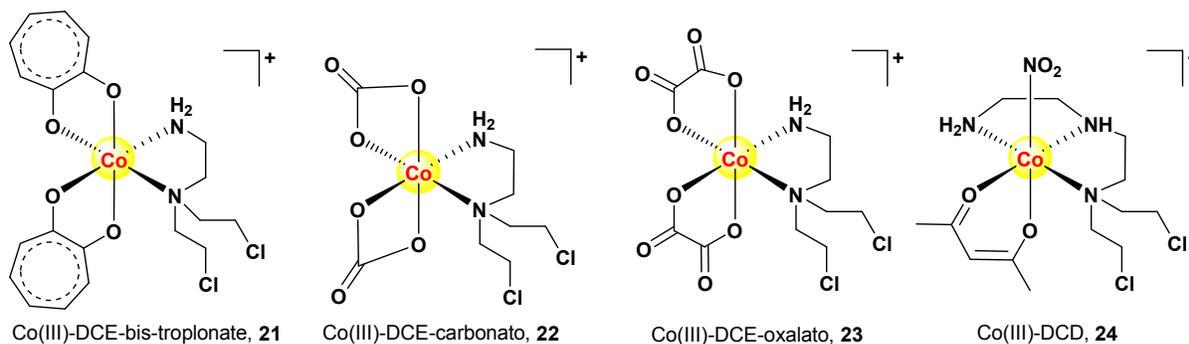


Figure 6. Chemical structures of rationally designed hypoxia active Co(III) prodrugs. The complexes displayed reasonable selectivity for cells grown in hypoxic conditions over those cultured in oxic conditions.

Cobalt(III) complexes as chaperones for bioactive ligands

Hambley and co-workers exploited the electrochemical properties of Co(III) complexes to selectively deliver matrix metalloproteinase (MMP) inhibitors to hypoxic cells.⁸⁹ MMPs are a family of zinc-dependent peptidases which have been implicated in cancer progression and other diseases.⁹⁰ MMPs breakdown the extracellular matrix (ECM) of cancer cells in primary tumour sites, facilitating cancer cell migration and metastasis.⁹¹ Although several exogenous MMP inhibitors have been developed to curb metastasis in cancer patients, none have been clinically approved (the only FDA-approved MMP inhibitor is doxycycline, which is used to treat periodontitis).⁹² A major shortcoming in the structural design of some of the MMP inhibitors developed to date (like marimastat and batimastat) is the exposed hydroxamic acid group. Indeed, the poor oral bioavailability of these inhibitors in clinical trials is thought to result from non-specific reactions between the hydroxamic acid group and biomolecules.⁹³ Hambley *et al.* elegantly overcame this problem by coupling MMP inhibitors to Co(III)-tris(2-methylpyridyl)amine (TPA) via the hydroxamic acid moiety.⁸⁹ This enabled deactivation of the MMP inhibitors in well oxygenated regions, and selective release in hypoxic sites (upon reduction of the inert Co(III) complex to the more reactive Co(II) complex). Initial electrochemical studies with prototypical systems containing simple alkyl and aryl hydroxamic acid ligands (**25a-c**), established that Co(III)-TPA was a suitable transporter of hydroxamic acid-containing compounds to hypoxic sites.⁹⁴ Following these results, marimastat, a potent MMP inhibitor, was attached to Co(III)-TPA via the two oxygen atoms on the hydroxamic acid moiety (**25d**) (**Figure 7**). This complex, **25d** suppressed 4T1.2 tumour growth in mice to a better extent than free marimastat. However a reproducible biochemical assay showed that **25d** (and free marimastat) potentiated rather than reduced metastasis. This was tentatively attributed to the failure of primary tumours in the control group to reach an appropriate size.⁸⁹ In light of this seemingly conflicting *in vivo* data, the authors suggested that further experiments with longer administration times were needed to gauge the true efficacy of **25d**. More recent studies involving Co(III)-TPA complexes, **25a-c** showed that both the hydroxamate and hydroximate forms could be isolated.⁹⁵ The reduction potential of the two protonated states was markedly different, and thus the ability of the complexes to release the bound hydroxamic acid ligand was also different. The differing electrochemical properties of the hydroxamate and hydroximate forms could be manipulated to develop Co(III) systems capable of delivering bioactive ligands (containing hydroxamic acids) to acidic regions. Given that many tumours are acidic as well as hypoxic, this strategy could be extremely useful in the development of new tumour specific delivery systems.

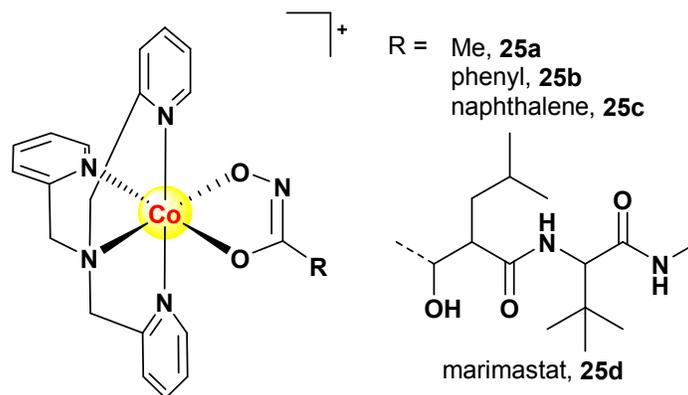


Figure 7. Chemical structure of Co(III)-tris(2-methylpyridyl)amine bound to various hydroxamic acid ligands including the MMP inhibitor, marimastat. The redox properties of Co(III)-TPA complexes are ideally suited to deliver bioactive ligands (containing hydroxamic acid functional groups) to hypoxic sites.

Apart from TPA, other tetradentate ligand systems have also been used to prepare Co(III) carriers for biologically active agents (**Figure 8**). Ware *et al.* showed that Co(III)-1,4,7,10-tetraazacyclododecane (cyclen) complexes were effective chaperones for cytotoxins such as 8-hydroxyquinoline (8-HQ) and azachloromethylbenzindoline (azaCBI, a DNA minor groove alkylator).^{72, 96, 97} Detailed mechanistic studies revealed that these systems (**26** and **27**) underwent efficient reduction (and cytotoxin release) in hypoxic cells through radiolytic and non-radiolytic pathways, upon exposure to ionising radiation. Moreover, **27** displayed selective potency (20-fold) towards colorectal adenocarcinoma cells (HT29) grown in hypoxic conditions over the same cells cultured in oxic conditions.⁷² Systematic modification of the azaCBI backbone yielded analogues with greater hypoxic selectivity.⁹⁸ Specifically, the incorporation of a dimethylaminoethanol group within the pyrroloquinoline ring (in the azaCBI unit) afforded **28**, with 41- and 31-fold hypoxic selectivity in ovarian carcinoma (SKOV3) and lung carcinoma (A549) cells, respectively. In an effort to improve solubility and stability, 1,7-substituted cyclen ligands containing anionic sulfonato, phosphinato and carboxylato groups were utilised (**29a-c** and **30a-c**).⁹⁹ Biological evaluation of **29a-c** and **30a-c**, revealed that although solubility was somewhat improved, their stability (in cell culture media) and hypoxic selectivity was commensurate to the parent complexes (**26** and **27**).

Larger macrocyclic ligands such as 1,4,8,11-tetraazacyclotetradecane (cyclam), with cross-bridged nitrogen atoms have also been used to prepare Co(III) chaperones for azaCBI (**Figure 8, 31a-b**).¹⁰⁰ The bridging of opposing nitrogen atoms within cyclam was proposed to force the nitrogen lone pairs to align with the cobalt d-orbitals, ensuring that only complexes with cis geometry could be formed. This was predicted to stabilise the Co(III) ion over the Co(II) ion and thereby decrease the reduction potential. This approach was used to generate a series of hypoxia selective Co(III) chaperones. The most effective complex in this series, **31a** displayed 81-212-fold greater toxicity under hypoxia than 20% oxygen against a panel of human cancer cell lines.¹⁰⁰ Surprisingly, the cytotoxicity of this complex was not enhanced in cell lines overexpressing one-electron reductase, NADPH: cytochrome P450 oxidoreductase (POR), which is a well-known initiator of other hypoxia-activated prodrugs (based on nitroaromatics and quinones).¹⁰¹ A similar trend was also observed for the cyclen-bearing derivative, **27**. Given these results, the identity of the reductase or non-enzymatic reductant responsible for the reduction of these complexes remains unknown. One theory is that the lipophilicity of these complexes facilitates mitochondrial accumulation, leading to reduction by the mitochondrial electron transport chain.⁷² However no experimental proof for this mechanism has been reported. Animal studies with **31a** showed the complex did not kill hypoxic (radioresistant) cells in HT29 tumour xenografts to an appreciable level.¹⁰⁰

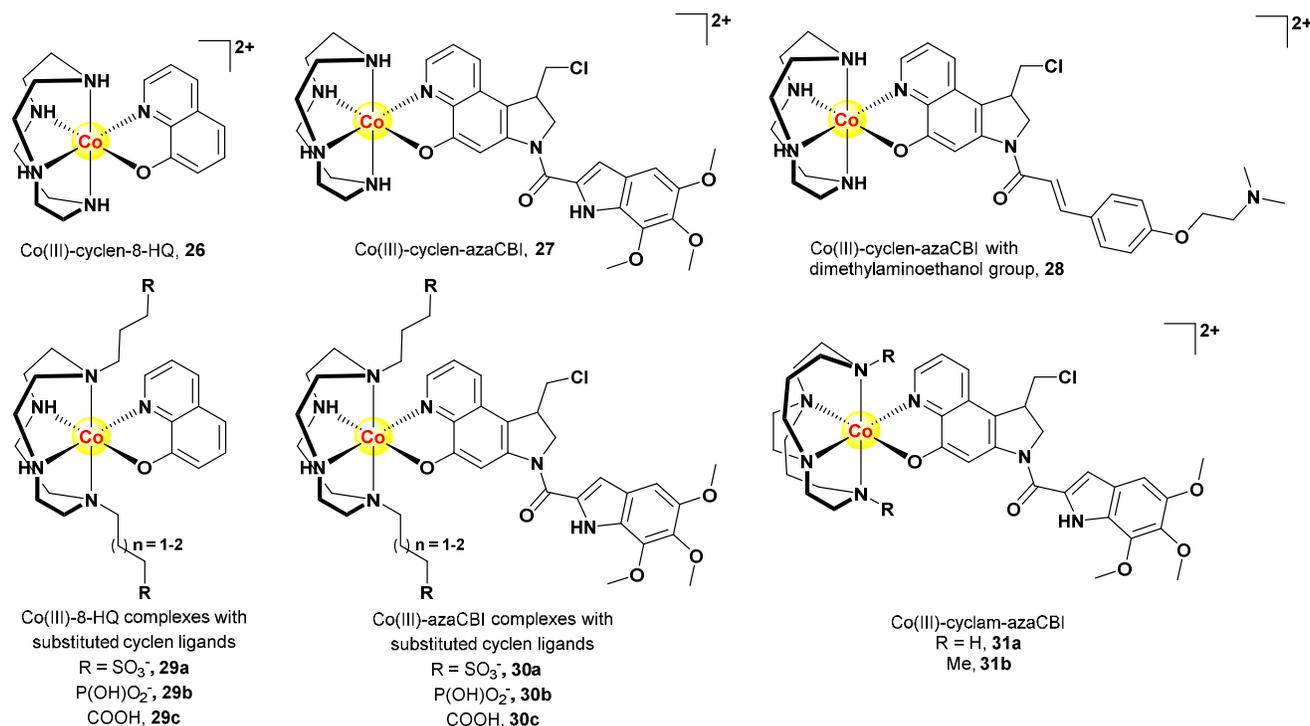


Figure 8. Chemical structures of Co(III) carriers with tetradentate ligand systems such as 1,4,7,10-tetraazacyclododecane (cyclen) and 1,4,8,11-tetraazacyclotetradecane (cyclam). These Co(III) systems effectively delivered cytotoxins to hypoxic cancer cells.

Cobalt(III) complexes for imaging hypoxic regions

As discussed previously, cobalt(III) complexes are thought to undergo bioreduction, leading to the release of a labile cobalt(II) complex and one or more bioactive ligands. However, the exact mechanism of activation remains elusive. Fluorophores have been utilised to shed light on the activation process. Cobalt(III) complexes containing one or two fluorophores that are themselves bioactive or mimic the binding profile of cytotoxins, and whose fluorescence is quenched upon coordination to cobalt, were investigated.^{22, 23} Upon reduction, the complexes were envisaged to release the fluorophores, resulting in a fluorescence turn-on. Co(III)-cyclam and Co(III)-TPA complexes containing the fluorescent ligand, coumarin-343 (c343H) (**32a**) and its hydroxamic acid derivative, coumarin-343 hydroxamic acid (c343haH₂) (**33a**), were initially used to probe the activation process (**Figure 9**).¹⁰² In the bound state, the inherent fluorescence of the coumarin ligands was quenched. The addition of biologically relevant reductants, ascorbic acid and glutathione led to partial fluorescence turn-on, indicative of the fluorophores being released by ligand exchange rather than Co(III) reduction. This was supported by the fact the reduction potential of the reductants used (+58 mV for ascorbic acid and -340 mV for glutathione) was not suited to reduce **32a** (-383 mV vs. NHE) or **33a** (-1334 mV vs. NHE). Furthermore, time-course fluorescence studies showed that the rate of fluorescence turn-on in the presence of ascorbic acid under oxygenated and deoxygenated conditions was comparable, consistent with ligand exchange as the mechanism of ligand release rather than redox cycling/ back-oxidation (*vide supra*). In vitro studies with ovarian carcinoma cells (A2780) revealed that the cytotoxicity of the **32a** and **33a** correlated with their cellular uptake and intracellular distribution.

Bryce and co-workers developed a series of Co(III) complexes capable of sensing hypoxic regions within an in vitro tumour model.¹⁰³ The most effective complex in this series comprised of a Co(III)-cyclam core and two axially bound anthraquinone-2-carboxylic acid (AQ2CH) ligands (**32b**). Unlike **32a**, the fluorescence intensity of **32b** only increased in the presence of sodium ascorbate under deoxygenated conditions, suggesting that ligand release was hypoxia selective, and mediated by reduction. Under oxygenated conditions, no fluorescence turn-on was observed implying that, in this case, re-oxidation competed with

ligand release. Confocal microscopy studies with an elaborate multicellular spheroid model consisting of colon carcinoma cells (DLD-1) expressing the photo-convertible EosFP green fluorescent protein under the control of the hypoxia response element (HRE), unambiguously showed that **32b** was able to penetrate deep into spheroids and preferentially release the fluorescent AQ2CH ligands under hypoxic conditions. Later the same group used carboxylic acid functionalised Co(III)-TPA complexes containing c343haH₂ (**33b**) to detect hypoxic and acidic regions within tumour spheroids (made up of DLD-1 cells).¹⁰⁴ More recently a series of Co(III)-TPA complexes with fluorescent curcumin ligands were employed to visualise hypoxic regions within DLD-1 spheroids (**34a-c**).¹⁰⁵ Fluorescent studies in the presence of reducing agents, and X-ray absorption (XANES) studies in DLD-1 cells suggested that the curcumin ligands were released by bioreductive activation. Fluorescence lifetime imaging (FILM) studies in solid tumour models showed that complexation of the curcumin ligands to Co(III)-TPA enabled better deliver. The cobalt chaperones, **34a-c** uniformly delivered the curcumin ligands throughout the tumour model whereas free curcumin accumulated in the outer edges.

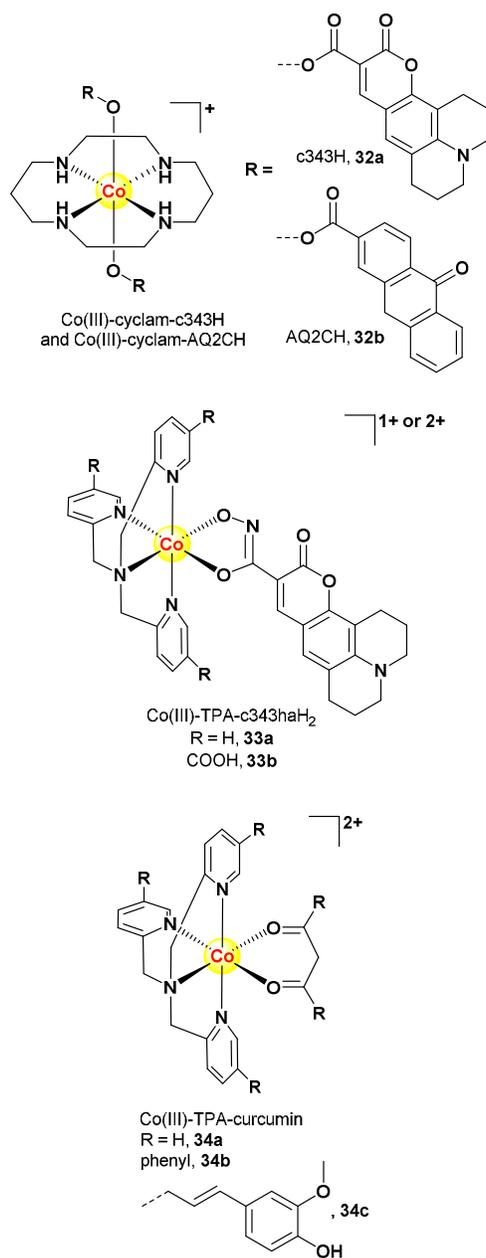


Figure 9. Chemical structures of Co(III)-TPA and Co(III)-cyclam complexes containing one or two fluorophores. These molecular systems were used to provide insight into the mechanism of activation and to image hypoxic regions within three-dimensional cell culture models.

Cobalamin bioconjugates for targeted delivery

Rapidly proliferating cells have a higher requirement for cobalt-containing cobalamin than non-transformed cells. Several organometallic cobalamin-drug bioconjugates have been prepared to exploit this dependence, and thus selectively target cancer cells (**Figure 10**). In general, the bioconjugates consist of a cytotoxic drug tethered to cobalamin via the β -axial position (mostly through a Co-C bond). The bioconjugates are extracellularly inert, but once internalised by receptor mediated endocytosis, undergo activation by β -axial bond cleavage, releasing the drug in its active form. Grissom *et al.* developed a cobalamin-chlorambucil bioconjugate (**35**) capable of killing leukemia cells (HL-60) to the same extent as free chlorambucil.¹⁰⁶ The toxicity of this bioconjugate was attenuated in the presence of excess cobalamin, proving that the cobalamin moiety was responsible for cell uptake. The same group also developed a cobalamin bioconjugate with colchicine (**36**), a tubulin-targeting chemotherapeutic drug.¹⁰⁷ Colchicine was attached to the cobalt centre via an acid labile hydrazone linker, enabling pH-dependent release. This bioconjugate exhibited nano-molar toxicity towards breast, brain, and melanoma cancer cells, however, the IC₅₀ values were 10-fold higher than free colchicine.

Cyanocobalamin (**37**) has been used to deliver cytostatic platinum compounds such as cisplatin to breast and ovarian cancer cells (MCF-7 and A2780).^{108, 109} The platinum compounds were fused to cyanocobalamin through a Pt-cyanide bond (**38a-c**). Biological studies showed that the platinum agents were released by reductive adenosylation upon cell entry. The cytotoxicity of the platinum-cyanocobalamin bioconjugates against breast and ovarian cancer cells were significantly lower than free cisplatin.¹⁰⁸ This was attributed to low receptor-mediated uptake of the bioconjugates. Cobalamin has also been used to transport small gaseous molecules of therapeutic significance. For example, nitric oxide (NO) was transported into cancer cells in the form of nitrosylcobalamin (**39**), a cobalamin derivative with NO fused to the β -axial position. Mechanistic studies showed that nitrosylcobalamin released NO upon cell entry, consequently inducing cell metabolism inhibition, DNA damage, and apoptosis.¹¹⁰ Extensive cytotoxicity studies (using twenty two tumour and two non-cancerous cell lines) showed that nitrosylcobalamin preferentially killed cancer cells over normal cells. In vivo studies in dogs with inoperable thyroid carcinoma, malignant peripheral nerve sheath tumour, apocrine gland adenocarcinoma, and spinal meningioma, proved very promising.¹¹¹ All of the treated dogs showed marked responses to therapy, with tumour reductions ranging from 43% to 77%. Remarkably, in one case, total remission was observed after 6 months of therapy. Given the encouraging in vivo data on nitrosylcobalamin, human trials are expected to follow. Cobalamin has also been coupled with carbon monoxide (CO)-releasing molecules (CORMs) for intracellular CO delivery.¹¹² This methodology has been employed to prevent ischemia-reperfusion injury in cultured cardiomyocytes, however, as far as we know it has not been applied to cancer related models.

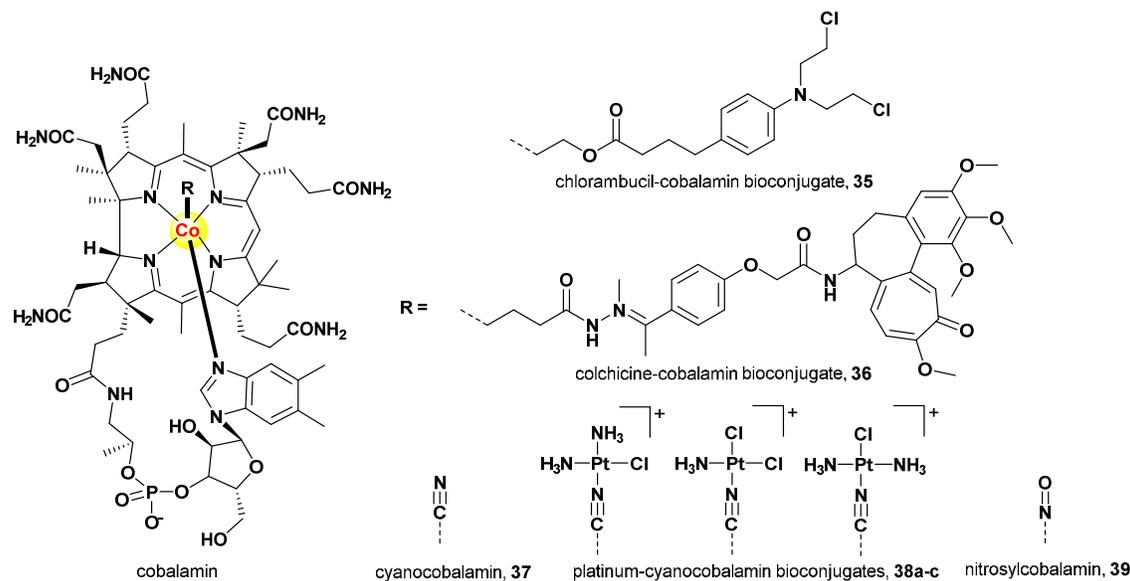


Figure 10. Chemical structures of cobalamin-drug bioconjugates. Cobalamin effectively delivered biologically active agents such as cisplatin, chlorambucil, and colchicine, to rapidly proliferating cancer cells. Nitrosylcobalamin, a cobalamin derivative with NO fused to the β -axial position, produced therapeutically appealing data.

Conclusion and outlook

Cobalt complexes have proven to be very promising anticancer agents worthy of further investigation. The diverse physiochemical properties of cobalt have enabled the preparation of several complexes with different cytotoxic modes of action. The role of cobalt in the therapeutic effect induced by these compounds is wide-ranging. For instance, the incorporation of cobalt-carbonyl clusters into NSAIDs directly enhances their cytotoxicity (probably through cobalt-biomolecule interactions), whereas the addition of cobalamin to small gaseous molecules of therapeutic potential improves their uptake into cancer cells (probably through receptor mediated endocytosis). The favourable redox properties of cobalt under physiological conditions have enabled the delivery of bioactive ligands and fluorophores to hypoxic and/or acidic cancer cells. The latter has facilitated detection of hypoxic cells in three-dimensional cell culture models. Further modifications and optimisations of these complexes could yield unprecedented strategies to treat and detect hypoxic tumours in hard to reach regions within the body.

Acknowledgements

We are thankful to Dr. Simon Teague for his suggestions and insight. K.S. is supported by a Leverhulme Early Career Fellowship (ECF-2014-178).

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