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Role of cell cycle events and apoptosis in mediating the anti-cancer activity of a silver(I) complex of 4-hydroxy-3-nitro-coumarin-bis(phenanthroline) in human malignant cancer cells

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ABSTRACT

The central objective of the current study was to investigate the potential *in vitro* anti-proliferative effect of 4-hydroxy-3-nitro-coumarin (hncH), and the mixed-ligand silver (I) complex of 4-oxy-3-nitro-coumarin-bis (phenanthroline), [Ag(hnc)(phen)$_2$] using four human-derived model cell lines. In addition, selected mechanistic studies were carried out using the most sensitive of the four cell lines. Results obtained show that the complex could decrease the proliferation of all four cell lines including neoplastic renal and hepatic, namely A-498 and HepG$_2$ cells, respectively, along with two non-neoplastic renal and hepatic cell lines, HK-2 and Chang, respectively. Furthermore, non-neoplastic hepatic cells (Chang) appeared to be less sensitive to the effect of the complex, but this effect was not replicated in the non-neoplastic renal (HK-2) cells. Based on IC$_{50}$ values [Ag(hnc)(phen)$_2$] was shown to be almost four times more potent than cisplatin, using HepG$_2$ cells. In addition, the observed anti-proliferative effect was shown to be both dose- and time-dependent. Furthermore, the complex was shown to decrease DNA synthesis, but did not intercalate with it. Moreover, there was no evidence that *P*-glycoprotein-mediated multi-drug resistance was likely to decrease anti-proliferative activity. Cytological stains, analysis of genomic DNA, and biochemical assays [caspase-3 and -9 and cleaved poly(ADP-ribose)-polymerase protein] showed that cell death appeared to result from apoptosis, with the possibility of secondary necrosis. Additionally, flow cytometric analysis showed that the complex functioned through an alteration in cell cycle progression. Taken together, [Ag(hnc)(phen)$_2$] has been shown to be a more potent anti-proliferative agent than cisplatin, capable of altering key biochemical events leading to cell death. Additional mechanistic studies are underway to probe more fully its mechanism of action.

1. Introduction

There have been numerous reports where metal-based drugs have successfully been used in the detection and treatment of a variety of diseases. These include cisplatin in cancer chemotherapy, ferrochloroquine in malaria, vanadium(IV) in the control of insulin, and gadolinium-coordinated contrast agents for magnetic resonance imaging (Fricker, 1994; Harpstrite et al., 2007). Platinum-based compounds have proven to be effective in the systemic treatment of cancer. Furthermore, only a few other transition metals, including ruthenium and gallium, have been extensively investigated for their potential anti-cancer activity (Galanski et al., 2003; Brabec and Novakova, 2006; Heffeter et al., 2006). Probably the best known metal-based anti-cancer drug is cisplatin [*cis*-diamminedichloroplatinum(II)]. It has been used widely in the treatment of cancers, especially testicular cancer, with a 70–90% cure rate. Cisplatin has been shown to bind to DNA following displacement of its chloride ions by hydroxyl groups. Covalent cross-linking leads to the formation of major adducts, which results in an inhibition of DNA replication and transcription (Zhang and Lippard, 2003). Furthermore, when combined with other drugs, cisplatin has successfully been used to treat brain, ovarian, bladder, and breast cancer (Marzano et al., 2002). The clinical success of cisplatin is limited by its significant side effects, such as nausea, vomiting, severe nephrotoxicity, and genotoxicity, along with resistance (Marzano et al., 2002; Sastry and Kellie, 2005). These limitations have stimulated a search for other transition metal complexes which are as or more effective, but with lesser side effects.

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Ruthenium complexes have attracted significant attention as building blocks for novel anti-cancer agents. These have been regarded as promising alternatives to platinum complexes (Hartinger et al., 2006; Tan et al., 2008). To date these complexes have been shown to offer potential over platinum-based compounds due to reduced toxicity and a novel mechanism of action, along with an absence of cross resistance (reviewed by Brabec and Novakova, 2006). Two structurally similar ruthenium(III) coordination compounds called KP1019 and NAMI-A have completed phase I clinical trial as anti-tumour agents and have been shown to be active against platinum-resistant malignancies including colorectal and pulmonary metastasis of solid tumours (Bouma et al., 2002; Bacac et al., 2004; Hartinger et al., 2006). The cellular target(s) of anti-tumour ruthenium complexes have not yet been fully identified. However, it has been speculated that their activity may be related to their ability to bind to DNA. In 2008, Lui et al. studied the in vitro anti-tumour activity of ruthenium(II) polypyridyl complexes. They showed significant activity with IC_{50} values in the micromolar range. In addition, they postulated that DNA binding was central to understanding their mechanism of action.

Recently, Heffeter et al. (2006) investigated the in vitro anti-tumour properties of a new lanthanum complex [tris(1,10-phenanthroline)lanthanum(III) trithiocyanate (KP772)] using a panel of tumour cells. Results showed IC_{50} values in the low micromolar range. These researchers showed that KP772 could induce morphological and biochemical changes, all of which are consistent with the induction of apoptotic cell death. In addition, this agent was shown not to target DNA, although minor induction of cross-linking was observed. Li et al. (2006) synthesised two lanthanum(III) complexes containing 2-methylene-1,10-phenanthroline units bridged by aliphatic diamines, and determined their in vitro anti-tumour activity. Results obtained showed that the complexes were more active than either the ligand or the salt, with DNA intercalation pivotal to mediating their activity.

Although epidemiological studies have shown that chromium is an inorganic carcinogen, chromium(III) salts have been shown to be non-carcinogenic due to their ability to cross the plasma membrane (Balamurugan et al., 2004). These researchers showed that chromium (III) complexes containing 1,10-phenanthroline (phen) showed significant in vitro anti-tumour activity in the micromolar range. In addition, they showed that cell death occurred as a result of apoptosis, possibly as a result of the generation of reactive oxygen species. Furthermore, its anti-tumour activity appeared to be dependant on the induction of apoptotic cell death.

Coumarins comprise a very large class of compounds found throughout the plant kingdom (Murray et al., 1982; Egan et al., 1990). The bio-activity of coumarin and more complex related derivatives appears to be based on the coumarin nucleus (Kolodziej et al., 1997; Jimenez-Orozco et al., 1999; Finn et al., 2001, 2004a). Biological effects observed include anti-bacterial (Laurin et al., 1999), anti-thrombotic and vasodilatory (Hoult and Paya, 1996), anti-mutagenic (Pillai et al., 1999), lipoxygenase and cyclooxygenase inhibition (Kimura et al., 1985; Hoffmanova et al., 1998), and scavenging of reactive oxygen species, as well as anti-tumourigenic effects (Maucher et al., 1993; Sharma et al., 1994; Egan et al., 1997; Hayes et al., 1998; Finn et al., 2004a,b; Thati et al., 2007a,b). Since the late 1980's, a number of *in vivo* studies have investigated the possible use of coumarins in the treatment of renal cell carcinoma (Marshall et al., 1986, 1994). All of these studies have demonstrated a significant response rate following coumarin treatment alone or in combination therapy. The *in vitro* effects of coumarins on the growth of renal cell carcinoma-derived cell lines showed that coumarin and 7-hydroxycoumarin are potent cytotoxic and cytostatic agents (Marshall et al., 1994). Recent studies carried out in our laboratory compared the anti-proliferative capability of a series of natural and synthetic nitro and hydroxylated derivatives of coumarin, including 6-nitro-7-hydroxycoumarin and 7,8-dihydroxycoumarin, using both renal adenocarcinoma and malignant melanoma cell lines. These compounds were shown to be potent cytotoxic agents, capable of killing cancer cells by modulation of key biochemical pathways such as mitogen-activated protein kinases (Finn et al., 2004a,b, 2005a,b).

1,10-Phenanthroline (phen) and its substituted derivatives, both in the metal-free state and as ligands co-ordinated to transition metals, have been shown to disturb the functioning of a wide variety of biological systems (Butler et al., 1969). Furthermore, when metal-free *N,N'*-chelating bases are found to be bioactive it is usually assumed that the sequestering of trace metals *in situ* is involved, and that the resulting metal complexes are the active species (MacLeod, 1952; Dwyer et al., 1969). Previous work has shown that the metal-phenanthroline complexes, namely $[Cu(phen)_2(mal)]\cdot 2H_2O$, $[Mn(phen)_2(mal)]\cdot 2H_2O$, and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ ($malH_2$ = malonic acid), could inhibit growth of the fungal pathogen *Candida albicans* by around 95% at a concentration range of 1.25–5.0 μg/ml (McCann et al., 2000; Coyle et al., 2003).

A number of reports have shown that metal-based derivatives of phen were capable of killing cancer cells in vitro. Specifically, Deegan et al. (2007) showed that $Cu(phen)_2(mal)]\cdot 2H_2O$ was a potent in vitro anti-proliferative agent, while Heffeter et al. (2006) showed that a lanthanium complex of phen, namely [tris(1,10-phenanthroline) lanthanium(III)trithiocyanate] could induce apoptosis in human-derived cell lines. In both cases the concentrations used were in the low micromolar range, and over short incubation times. Other researchers have shown that $[Cu(phen)_2]^{2+}$ is a biologically active metal–phenanthroline complex (Samuni et al., 1981; Wijker and Lafleur, 1999). This agent has been shown to promote hydroxyl radical formation from molecular oxygen by redox-cycling and could therefore be considered suitable for stimulating the production of reactive oxygen species. Transition metal cations such as Cu(II) and Fe(II) bind to negatively-charged DNA and have been shown to play an important role in the local formation of OH radicals (Samuni et al., 1981; Wijker and Lafleur 1999). One of the consequences of high copper levels in the body has been shown to be an increase in the rate of radical formation leading to oxidative damage, resulting in disruption of lipid bilayers due to oxidation and cleavage of vulnerable unsaturated fatty acid residues of phospholipids (Linder, 2001). Alterations in protein function have also been shown to be promoted through oxidation of thiol and possibly amino groups. In addition, researchers have suggested that gene expression may be altered due to oxidation of guanosine and adenosine residues in nucleic acids, along with an alteration in transcription factor/growth factor activities (Linder, 2001). Tsang et al. (1996) reported that incubation of a human hepatic cell line (Hep-G2) with $[Cu(phen)_2]^{2+}$ resulted in internucleosomal DNA fragmentation. Zhou et al. (2002) also reported G_1-specific apoptosis in a liver carcinoma cell line (Bel-7402), caused by $[Cu(phen)_2]^{2+}$. Additionally, this complex was shown to up-regulate the DNA-binding activity of p53, a molecule know to be pivotal in the regulation of cell progression, cell survival, and apoptosis (Verhaegh et al., 1997).

Interest in metal–coumarin complexes has arisen from the search for novel lead compounds, along with the desire to improve the pharmacological profile of established anti-neoplastic agents. Kokotos et al. (1997) synthesised a number of amino-coumarin–platinum(II) complexes and evaluated their *in vitro* anti-proliferative activity using a colonic carcinoma cell line (Caco-2). They screened a number of coumarins with an amino group at position six or seven and methyl groups at various positions around the coumarin nucleus. These researchers found that the most potent platinum–coumarin complex had functional groups attached at position six. Additionally, Kostova et al. (2001) studied the effects of coumarin complexed to zirconium. Here again it was found that metal–coumarin complexes were more active on their own. In 2002, Manalov et al. investigated the *in vitro* cytotoxicity of a number of coumarin complexes with the late lanthanoid transition metal, cerium, using both Burkitt lymphoma