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Chemico-Biological Interactions 164 (2006) 115–125
Chemico-Biological Interactions
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# *In vitro* anti-tumour effect of 1,10-phenanthroline-5,6-dione (phendione), $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$ and $[Ag(phendione)_2]ClO_4$ using human epithelial cell lines

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Received 31 March 2006; received in revised form 11 August 2006; accepted 17 August 2006
Available online 16 September 2006

**Abstract**

The anti-cancer chemotherapeutic potential of 1,10-phenanthroline-5,6-dione (phendione), $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$ and $[Ag(phendione)_2]ClO_4$ were determined using four human cells lines, i.e. two neoplastic (A-498 and Hep-G2) and two non-neoplastic (CHANG and HK-2). All of the phendione derivatives induced a concentration-dependant decrease in the viability of the four cell lines, with $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$ displaying greatest activity. In comparative studies, $IC_{50}$ values obtained with the two neoplastic cell lines showed a cytotoxic response which was between 3 and 35 times greater than that observed for the metal-based anti-cancer agent, cisplatin. Furthermore, metal–phendione complexes, rather than simple solvated metal ions, were responsible for the observed cytotoxicity. Despite the high level of potency associated with these compounds they did not display an apparent cyto-selective profile, as they reduced the viability of both neoplastic and non-neoplastic cells. However, selected mechanistic studies showed that phendione and its metal complexes inhibited DNA synthesis which did not appear to be mediated through intercalation. Ames testing highlighted that all three compounds and their phase I metabolites were non-mutagenic, unlike cisplatin. Taken together, these results suggest that phendione and its Cu(II) and Ag(I) complexes may be capable of acting as highly effective anti-cancer therapies, which with careful administration could provide very potent and effective alternatives to cisplatin.


*Keywords:* 1,10-Phenanthroline-5,6-dione; Transitional metal complexes; DNA inhibition; Anti-cancer potential

## 1. Introduction

Recently there have been a number of reports highlighting the use of transition metal complexes as anti-cancer agents [1–3]. Probably the best known of these is cisplatin [*cis*-diaminedichloroplatinum(II)]. It has been widely used in the treatment of a variety of cancers, especially testicular cancer, with a 70–90% cure rate. When combined with other drugs, it has been used successfully to treat brain, ovarian, bladder and breast cancer [4]. The clinical success of cisplatin is limited by its significant side effects, such as nausea, vomiting and severe nephrotoxicity [4]. Additionally, acquired

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doi:10.1016/j.cbi.2006.08.025

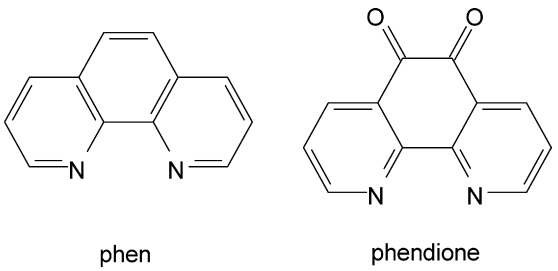



Fig. 1. Structure of 1,10-phenanthroline (phen) and 1,10-phenanthroline-5,6-dione (phendione).

resistance has served to limit the widespread use of cisplatin [5–7]. The employment of cisplatin and related platinum complexes as anti-cancer agents has stimulated the search for other active transition metal complexes which are as effective, but with lesser side effects [5–7].

1,10-Phenanthroline (phen) (Fig. 1) and substituted derivatives, both in the metal-free state and as ligands co-ordinated to transition metals, disturb the functioning of a wide variety of biological systems [8]. 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 [9,10]. Previous work has shown that metal–phen complexes $[Cu(phen)_2(mal)]\cdot 2H_2O$, $[Mn(phen)_2(mal)]\cdot 2H_2O$ and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ ($malH_2$ = malonic acid) inhibit growth of the fungal pathogen *Candida albicans* by around 95% in a concentration range 1.25–5.0 μg/ml [11,12].

One of the most biologically active of the metal–phen complexes is $[Cu(phen)_2]^{2+}$. 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 $^{\bullet}OH$ radicals [13,14]. One of the consequences of high copper levels in the body is an increase in the rate of radical formation leading to oxidative damage [15]. This leads to a disruption of lipid bilayers due to oxidation and cleavage of vulnerable unsaturated fatty acid residues of phospholipids. Alterations in protein function are also promoted through oxidation of thiol and possibly amino groups. Gene expression may also be altered due to oxidation of guanosine and adenosine residues in nucleic acids, or altered transcription factor/growth factor activities [15]. Tsang et al. [16] reported that incubation of a human hepatic cell line (Hep-G2) with $[Cu(phen)_2]^{2+}$ resulted in internucleosomal DNA fragmentation, a hallmark of apoptosis. Zhou et al. [17] also reported G1-specific apoptosis in a liver carcinoma cell line (Bel-7402), caused by $[Cu(phen)_2]^{2+}$. Additionally, this complex was also shown to up-regulate DNA-binding activity of p53, a pivotal molecule in the regulation of cell progression, cell survival and apoptosis [18].

1,10-Phenanthroline-5,6-dione (phendione) is a chelate ligand containing an *o*-quinoid moiety which is considered to have many interesting biological properties. Due to its redox activity, phendione both in its metal-free state and in complexes with transition metals, such as ruthenium, cobalt, and osmium show strong electrocatalytic activity in the oxidation of NADPH [19–22]. Yokoyama et al. [22] have shown that ruthenium complexes of phendione, particularly its quinine moiety, can chemically modify proteins, such as cytochrome *c*, resulting in increased reactivity suggesting that these metal complexes may act as chemical modifiers. More recently, Ghosh et al. [23] has studied the ability of cobalt(III) complexes of phen and phendione to interact with DNA using a variety of assays including covalent binding assays, viscosity measurements' and competitive binding fluorescence measurements. These researchers showed that the complexes could intercalate within the base pairs of DNA, along with cleaving plasmid DNA upon irradiation under aerobic conditions. Furthermore, molecular modelling was used to show that they could fit into the major groove without disrupting the helical structure of B-DNA. Based on these findings, these researchers suggest that derivatives of phendione may display anti-cancer activity, and studies are currently underway to prove this theory.

The primary aim of the current study was to evaluate the cancer chemotherapeutic potential of 1,10-phenanthroline-5,6-dione, $[Cu(phendione)_3](ClO_4)_2\cdot 4H_2O$ and $[Ag(phendione)_2]ClO_4$, using four human-derived cancer cell lines. In order to ascertain the likely effect of these agents on non-neoplastic cells, we decided to include two cell lines, both derived from normal human cells. These two non-neoplastic cells lines were chosen as they provide the closest match with the neoplastic cell lines currently commercially available. Furthermore, in order to illustrate that the cytotoxic effect observed was due to the complexes rather than the free metal ions, the anti-tumour activities of the simple Cu(II) and Ag(I) salts, $Cu(ClO_4)_2$ and $AgClO_4$ were also determined. In addition, the relative potency of these test agents was determined by the inclusion of one of the best known and most biologically active metal-based anti-cancer agents,

cisplatin. Aspects of the molecular mechanisms underlying the anti-proliferative response were probed by investigating the role of the complexes in mediating DNA synthesis and intercalation. This work presented here represents the first thorough assessment of the potential application of phendione, $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$, and $[Ag(phendione)_2]ClO_4$ as novel therapeutic agents for the treatment of cancer.

## 2. Materials and methods

### 2.1. Test compounds

Cisplatin, $Cu(ClO_4)_2 \cdot 6H_2O$, $AgClO_4$ and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Ltd. 1,10-Phenanthroline-5,6-dione (phendione) was made in accordance with the literature method [24]. $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$ was prepared using a slight modification of the method used by Liu et al. [25] in their synthesis of $[Cu(phendione)_3](ClO_4)_2 \cdot 2H_2O \cdot 2MeCN$ [26]. $[Ag(phendione)_2]ClO_4$ was made by the method specified by McCann et al. [26]. All cell culture reagents and media were purchased from Euroclone, UK, unless otherwise stated. Structure and purity of phendione and its metal-based complexes was confirmed by thin layer chromatography, infrared analysis, $^1H$ and $^{13}C$ NMR spectroscopy, along with microanalysis.

### 2.2. Model cell lines

A-498 (human kidney adenocarcinoma), HK-2 (human proximal tubular), CHANG (human hepatic) and Hep-G2 (human hepatocellular carcinoma) cells were purchased from the American Type Culture Collection, Manassas, USA. A-498, CHANG and Hep-G2 cells were maintained in Eagle's minimum essential medium (EMEM) with Earle's balanced salt solution (EBSS) containing 1.5 g/l sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal bovine serum (Sigma). HK-2 cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Hams F12 (50:50 v/v), supplemented with 2 mM L-glutamine, ITS (5 μg/ml bovine insulin, 5 μg/ml human transferrin and 5 ng/ml selenium), 36 ng/ml hydrocortisone, 4 pg/ml 3,3′,5-triiodo-L-thyronine, and 10 ng/ml epidermal growth factor. All cell lines were grown at 37 °C in a humidified atmosphere with 5% $CO_2$, and were in the exponential phase of growth at the time of inclusion in cytotoxicity assays.

### 2.3. Assessment of cytotoxicity, using MTT assay

Phendione, $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$, $[Ag(phendione)_2]ClO_4$, cisplatin and the simple metal salts $Cu(ClO_4)_2 \cdot 6H_2O$ and $AgClO_4$ were dissolved in DMSO, diluted in culture media and used to treat model cell lines for a period of 96 h. The maximum percentage of DMSO present in all wells was 0.2% (v/v). Cells were seeded in sterile 96-well flat-bottomed plates (Sarstedt) at a density of $5 \times 10^4$ cells $cm^{-3}$ and grown in 5% $CO_2$ at 37 °C. A miniaturised viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was carried out [27]. In metabolically active cells, MTT is reduced by the mitochondrial enzyme succinate dehydrogenase to form insoluble purple formazan crystals that are subsequently solubilised, and the optical density (OD) measured spectrophotometrically. Therefore, drug-treated cells were assayed by the addition of 20 μl of 5 mg/ml MTT in 0.1 M phosphate buffer saline (PBS), pH 7.4. Following incubation for 4 h at 37 °C, the overlying media was aspirated with a syringe and 100 μl of DMSO was added to dissolve the formazan crystals. Plates were agitated at high speed to ensure complete dissolution of crystals and OD was measured at 550 nm using an Anthos HT-II microtitreplate reader. Viability was expressed as a percentage of solvent-treated control cells. Each drug concentration had five replicates per assay and each experiment was carried out on at least three separate occasions. The $IC_{50}$ value was calculated for each drug and used as a parameter to compare the cytotoxicity of each test compound. Consequently, the $IC_{50}$ was defined as the drug concentration (μM) causing a 50% reduction in cellular viability.

### 2.4. DNA synthesis studies

The effect of phendione, $[Cu(phendione)_3](ClO_4)_2 \cdot 4H_2O$, and $[Ag(phendione)_2]ClO_4$ on DNA synthesis was determined using the 5-bromo-2-deoxyuridine (BrdU) colourimetric incorporation assay [29]. 100 μl of A-498 and Hep-G2 cells were seeded into 96-well plates to a density of $5 \times 10^4$ cells/ml and allowed to adhere overnight, after which test compound was incubated for 96 h prior to the addition of BrdU (10 μM), and incubated for a further 4 h at 37 °C. Following removal of media, cells were permeabilised and fixed by the addition of FixDenat™ solution (200 μl/well), and then incubated at room temperature for 30 min, after which reagent was removed. Incorporated BrdU was detected by the addition of anti-BrdU-POD antibody conjugate (100 μl/well) (Roche), followed by incubation at room

temperature for 2 h. Plates were washed three times with wash buffer (supplied by the manufacturer), followed by the addition of TMB substrate solution (100 μl/well) and incubation at room temperature for 30 min, while being protected from light. The reaction was then stopped by the addition of 1 M $H_2SO_4$ (25 μl/well). The OD of samples was measured on a Sunrise ELISA reader at 450 nm (reference wavelength 690 nm). Results for drug-treated cells were expressed as percentage BrdU incorporation (DNA synthesis) of solvent-treated control cells. Each drug concentration had five replicates per assay and each experiment was carried out on at least three separate occasions.

### 2.5. DNA binding studies

pGEM-3Z plasmid DNA was purified from *Escherichia coli* [strain JM 109 as previously cultured in LB broth (Oxoid), containing 50 μg/ml ampicillin] using a Qiagen isolation kit (Qiagen Ltd.). DNA purity and concentration was determined spectrophotometrically using A260/A280 spectrophotometric measurements. DNA concentration was adjusted to 1 μg/ml using 10 mM Tris–HCl, pH 7.5, containing 1 mM EDTA. Drug binding assays were carried out using phendione, metal–phendione complexes and the two simple metal salts, according to the method described by Lorcozio and Long [29]. Briefly, DNA was incubated for 2 h at 37 °C. Doxorubicin was employed as a positive control throughout. DNA was separated on a 1% (w/v) agarose gel in TBE (80 mM Tris–HCl, pH 8; 40 mM boric acid; 2 mM EDTA), and stained with ethidium bromide (5 μg/ml in TBE). Bands were visualised by irradiation at 300 nm and photographed using a Pharmaciae 3D imaging system.

### 2.6. Plate incorporation mutagenicity assay

A Standard Ames test was carried out using phendione, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$ at concentrations close to their respective $IC_{50}$ values (0–10 μM) as determined by MTT assay above. *Salmonella typimurium* tester strains TA98 and TA102 were used to detect possible mutation by both frame-shift and base-pair substitution, respectively. Additionally, the mutagenic potential of phase I metabolites of each of the three test phendione agents was determined by the inclusion of an S9 fraction isolated from rat hepatocytes, where the animal had previously been exposed to Aroclor 1254. The number of revertant colonies was determined and related to drug concentration [30].

### 2.7. Statistics

Statistical evaluation of the untreated control cells along with drug- and solvent-treated cells was calculated using one-way ANOVA (analysis of variance). A probability of 0.05 or less was deemed statistically significant. The following notation was used throughout, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, differences to control.

## 3. Results

The primary objective of the current study was to ascertain the anti-cancer potential of phendione and two of its metal-based complexes, namely $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$, using two pairs of cell lines, one of neoplastic and the second of non-neoplastic origin. In addition, key aspects of the mechanism of action of these agents were also determined.

### 3.1. Assessment of cytotoxicity

All test agents were initially incubated with four human-derived cell lines, two neoplastic (A-498 and Hep-G2) and two non-neoplastic cell lines (CHANG and HK-2) in order to determine their cytotoxic profile. Cellular viability was determined using MTT with an incubation period of 96 h. Data obtained for phendione and its metal complexes, along with the simple metal salts $AgClO_4$ and $Cu(ClO_4)_2{\cdot}6H_2O$ is presented in Figs. 2–4 and these graphs were used to calculate the $IC_{50}$ values presented in Table 1.

Figs. 2 and 3 clearly demonstrate that all of the test compounds, particularly phendione, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$, and $[Ag(phendione)_2]ClO_4$ produced a concentration-dependant cytotoxic response in all four cell lines. Furthermore, using the neoplastic-derived cell lines, the $IC_{50}$ values (Table 1) for the two phendione–metal complexes, and particularly $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$, were statistically lower than those obtained for metal-free phendione. This finding suggests that coordinated metal ions play a major role in mediating the potency of the complex. Additionally, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ is the most cytotoxic agent, and in all four cell lines. Furthermore, based on the $IC_{50}$ values presented in Table 1, it would appear that phendione and its two complexes are toxic to all four model cells lines, suggesting that these compounds are incapable of selectively killing cancer cells, while leaving normal cells viable. This effect was also observed for cisplatin with the two renal cell lines. Furthermore, in comparative cytotoxicity assays,

Table 1
Cytotoxic potential of phendione, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$, $[Ag(phendione)_2]ClO_4$, simple Cu(II) and Ag(I) perchlorate salts, along with cisplatin using human renal (HK-2 and A-498) and hepatic (CHANG and Hep-G2) cell lines following continuous incubation for 96 h and MTT assay

| Compound | HK-2 ($IC_{50}$ (μM) ± S.E.M.) | A-498 ($IC_{50}$ (μM) ± S.E.M.) | CHANG ($IC_{50}$ (μM) ± S.E.M.) | Hep-$G_2$ ($IC_{50}$ (μM) ± S.E.M.) |
|---|---|---|---|---|
| Phendione | 0.7 ± 0.08 | 4.2 ± 0.36 | 0.4 ± 0.01 | 1.4 ± 1.34 |
| $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ | 0.5 ± 0.14* | 0.88 ± 0.06* | 0.2 ± 0.02* | 0.78 ± 0.09* |
| $[Ag(phendione)_2]ClO_4$ | 0.8 ± 0.06* | 1.4 ± 0.47* | 0.3 ± 0.11* | 0.86 ± 0.87* |
| $Cu(ClO_4)_2{\cdot}6H_2O$ | 200 ± 10.5 | 973.3 ± 26.67 | >1000 | >1000 |
| $AgClO_4$ | 65 ± 2.0 | 44.4 ± 2.34 | 19 ± 1.8 | 7.6 ± 0.70 |
| Cisplatin | 18 ± 2.7 | 14.0 ± 1.00 | 45 ± 2.9 | 15.0 ± 2.65 |

A graph of viability (% of solvent-treated control cells) vs. drug concentration was used to calculate $IC_{50}$ values (μM). Results are representative of three independent experiments (mean ± S.E.M., $n = 5$).
* Statistically distinct from that of the parent ligand at $p < 0.05$.

$IC_{50}$ values for phendione and its complexes show cytotoxicities of between 3 and 35 times greater than that observed for cisplatin (Table 1). This finding is significant, as it suggests that phendione and its metal-based complexes may be an even more potent alternative to cisplatin.

### 3.2. Investigation of DNA as a possible molecular target

In an attempt to elucidate the key events responsible for the observed reduction in cellular viability, the effects on DNA synthesis were determined using BrdU incorporation assays. These assays were carried out following a 96 h drug treatment period. Results obtained suggest that phendione and the metal–phendione complexes cause a dose-dependant decrease in DNA synthesis (Fig. 5). Furthermore, phendione and $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ appeared to be the most potent agents across both neoplastic-derived model cell lines, a trend which was consistent with the cytotoxicity data obtained from MTT assays.

The planar nature of the phendione molecule might imply the possible involvement of intercalation in mediating the observed toxic response. Therefore, electrophoretic mobility shift assays were carried out, where DNA was incubated with test agents over a range of drug concentrations (1, 10 and 200 μM) and subsequently separated by electrophoresis. The results obtained (Fig. 6) clearly indicate that phendione and $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ did not alter the migration of any of the three forms of plasmid DNA (i.e. super-coiled, linear, or open-circular), unlike the positive control doxorubicin (Fig. 6). Similar results were obtained for $[Ag(phendione)_2]ClO_4$ (data not shown). These findings suggest that phendione and its Cu(II) and Ag(I) complexes do not intercalate DNA and thus presumably function by an alternative mechanism.

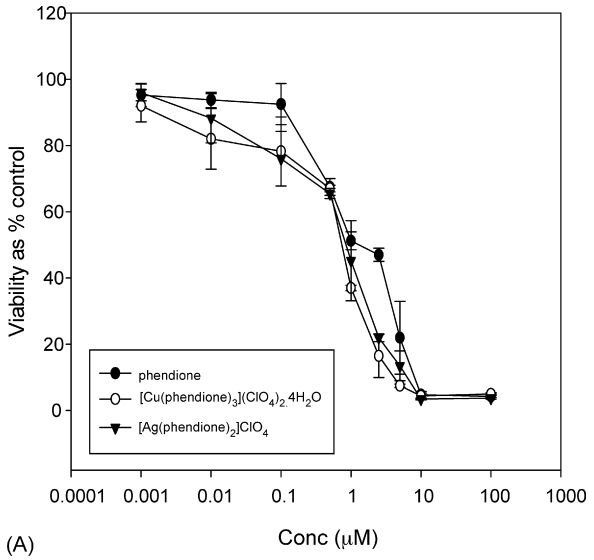
### 3.3. Investigation of mutagenic potential

The results obtained above suggest that phendione, and particularly $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$, are potent anti-cancer agents which function by inhibiting DNA synthesis. However, their usefulness would be limited if they also induced cellular mutations. Here, the Standard Ames test was employed to establish the mutagenic potential of the three phendione compounds along with their phase I metabolites. Additionally, the ability of the test agents to induce mutations by both frame-shift and base-pair substitution was determined by the inclusion of two distinct tester strains, namely TA98 and TA102. The results presented in Fig. 7 show that neither phendione, $[Ag(phendione)_2]ClO_4$, nor their phase I metabolites cause mutation by either frame-shift or base-pair substitution. Similar results were also obtained for $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ (data not shown).

Finally, results presented here confirm that phendione, and particularly $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$, are potent cytotoxic agents, capable of decreasing both cellular viability and DNA synthesis, but without the involvement of either intercalation or mutation. Taken together, our data suggest that these compounds, while they do not display an apparent cyto-selective nature, may offer an alternative to cisplatin in the treatment of human cancers.

## 4. Discussion

Phen and its copper complexes have previously been shown to exert a range of biological activities, such



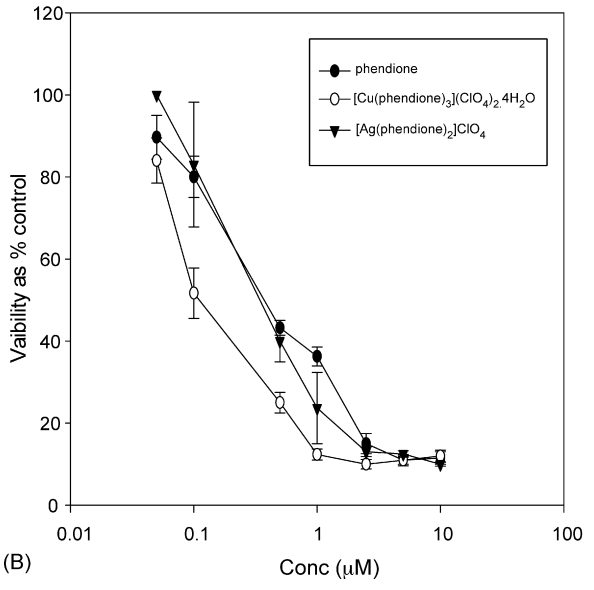
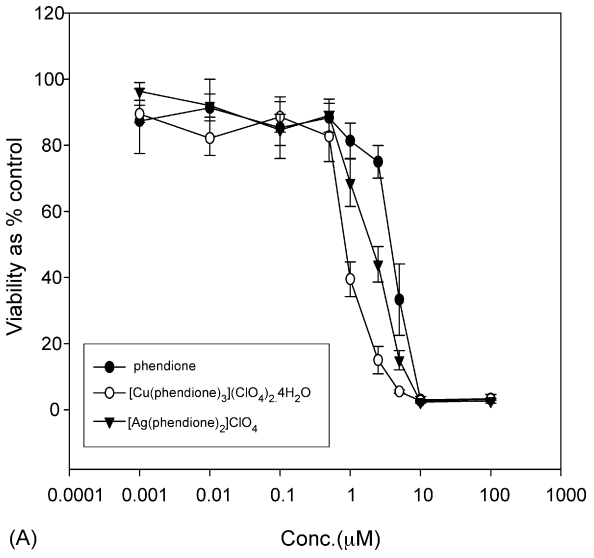




Fig. 2. Effects of phendione, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$ on the viability of (A) Hep-G2 and (B) CHANG cells. All compounds showed a concentration-dependant decrease in viability and in both cell lines, following 96 h incubation. Results are expressed as percentage viability of solvent-treated control cells. Bars indicate ±S.E.M., $n = 3$.



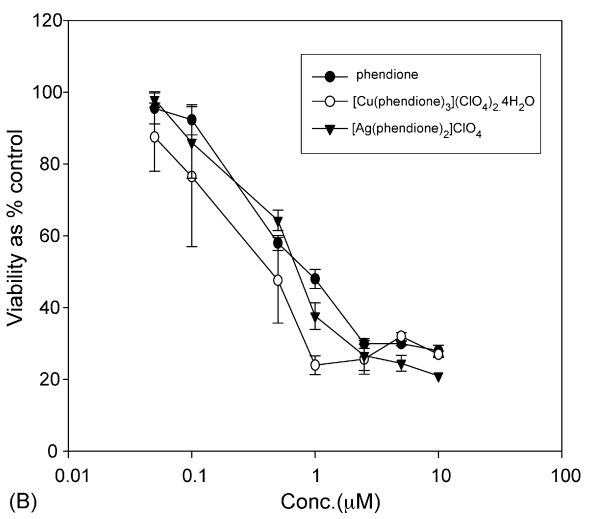




Fig. 3. Effects of phendione, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$ on the viability of (A) A-498 and (B) HK-2 cells. All compounds showed a concentration-dependant decrease in viability and both cell lines, following 96 h incubation. Results are expressed as percentage viability of solvent-treated control cells. Bars indicate ±S.E.M., $n = 3$.

as anti-tumour [31–33], anti-*Candida* [32,34], anti-mycobacterial [35] and anti-microbial effects [36]. Additionally, phendione has recently been shown to exert anti-fungal activity [26]. In this study, we attempted to identify the anti-cancer chemotherapeutic potential of phendione and two of its metal–phendione complexes, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$, along with characterising key aspects of their *in vitro* mode of action. However, given the scope of the current paper, this does not represent a thorough investigation of their mechanism of action. Additionally, in the current study, we acknowledge that there is the possibility that each of the effects observed here may be due to either the parent compound (i.e. phendione and two of its metal–phendione complexes) its metabolites or both, the latter being formed within the cells during the specified incubation periods. Additionally, in attempting to evaluate the cyto-selective nature of these test agents we attempted to select human model cell lines that provide the best possible means of comparison. However, it is

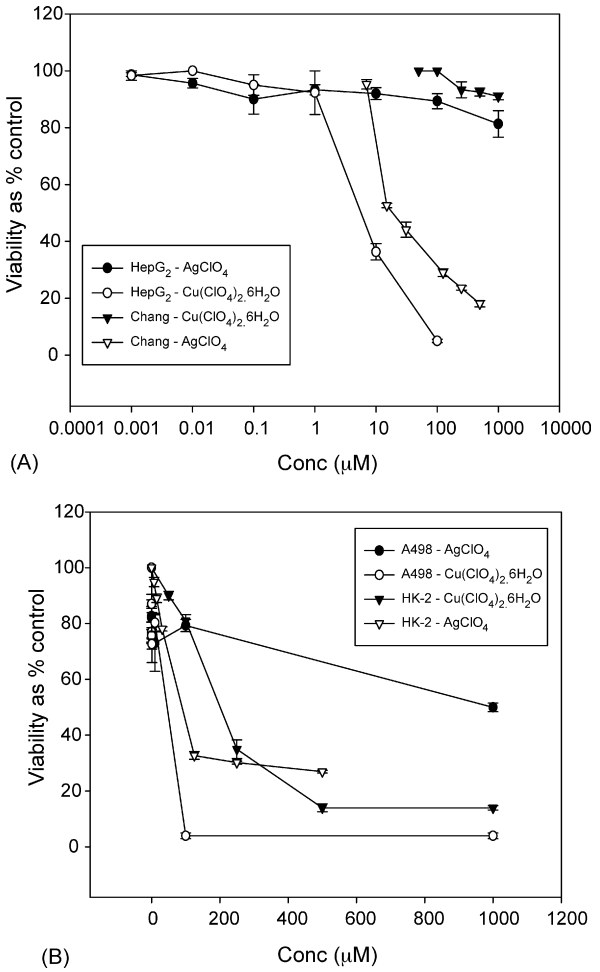



Fig. 4. Effects of the simple metal perchlorate salts, $Cu(ClO_4)_2{\cdot}6H_2O$ and $AgClO_4$, on the viability of (A) A-498 and HK-2 cells along with (B) Hep-G2 and CHANG cells. All salts promoted a decrease in viability following 96 h incubation. However, $AgClO_4$ appeared to be the most potent, and in both cell lines Results are expressed as percentage viability of solvent-treated control cells. Bars indicate ±S.E.M., $n = 3$.

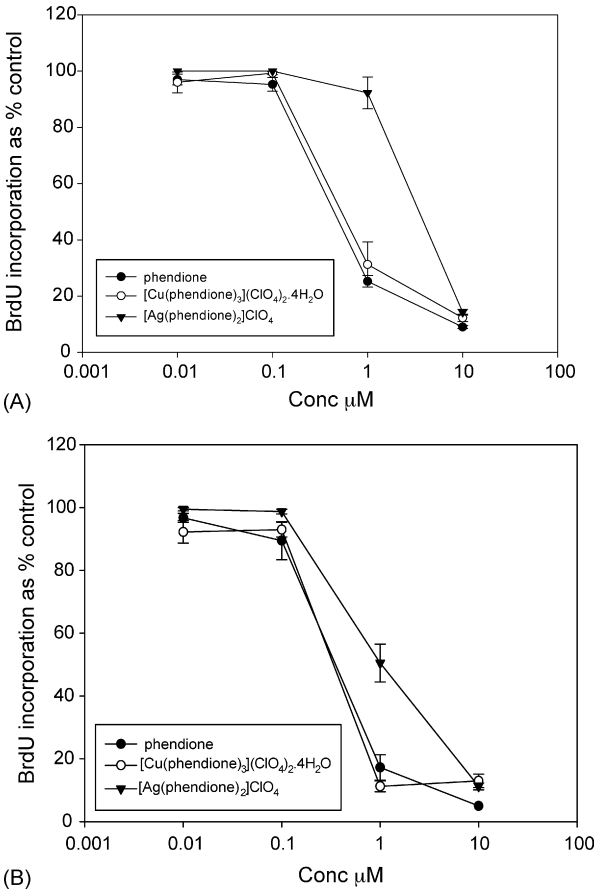



Fig. 5. Effects of phendione, $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$ on DNA synthesis using BrdU incorporation assays in (A) A-498 and (B) Hep-G2 cells, following continuous exposure for 96 h. All compounds produced a dose-dependant decrease in DNA synthesis. Bars indicate ±S.E.M., $n = 3$.

worth noting that the neoplastic and non-neoplastic cells are not identical, since, their media requirements and growth kinetics differ. Hence, there remains the possibility that these factors may contribute to the apparent non-selective nature of the test agents.

Cispatin is one of the best-known metal-based drugs currently in clinical use [4]. It has been shown to be highly effective against a variety of solid tumours. However, severe nephrotoxicity and resistance [5–7], which it exhibits and driven the search for compounds but with similar or enhanced potency and with significantly reduced side-effects. The mode of action of cisplatin has been studied extensively. It has been shown that on entering the cell, $Cl^-$ dissociates to leave a reactive complex which can react with water, which in turn reacts with DNA, forming inter- and intra-strand DNA cross-links. It is this reaction which leads to local denaturation of the DNA chain. In addition, cisplatin has been shown to cause mitochondrial damage, and it arrests, cell cycle in G phase, inhibits ATPase activity, and alters the cellular transport system, eventually leading to apoptosis, inflammation, and necrosis (reviewed by Ali and Moundhri [37]).

The central objective of the current paper is to determine the anti-cancer potential of phendione and two of its metal-based complexes, namely $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ and $[Ag(phendione)_2]ClO_4$, using two pairs of cell lines (one pair of neoplastic origin and the second non-neoplastic). It was felt that this method would allow us to clearly identify whether these agents were capable of selectively killing cancer cells, while leaving non-cancerous cells viable. Although this approach is relatively new in *in vitro* anti-cancer

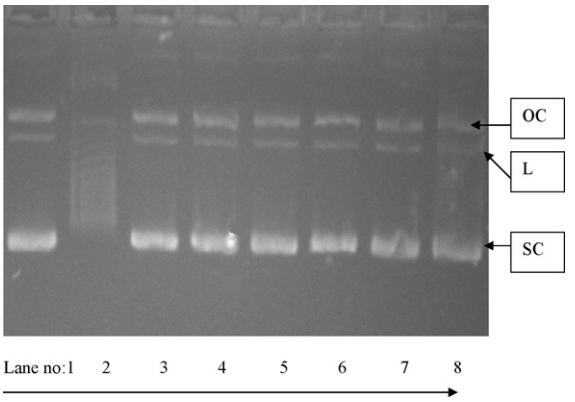



Fig. 6. Electrophoretic mobility shift assays were used to determine the effects of phendione and $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ on the migration of pGEM-3Z supercoiled plasmid DNA for 2 h at 37 °C, analysed by agarose electrophoresis and stained with ethidium bromide. Results indicate that neither compound inhibited migration of super coiled (SC), linear (L) or open circular (OC) forms of plasmid DNA, suggesting they do not intercalate DNA. Lane 1, negative control (pGEM-3Z DNA); lane 2, positive control (pGEM-3Z DNA and doxorubicin, 10 μM); lanes 3–5, phendione (pGEM-3Z DNA and phendione at 1, 10 and 200 μM); lanes 6–8 (pGEM-3Z DNA and $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ at 1, 10 and 200 μM). Similar results were obtained for $[Ag(phendione)_2]ClO_4$ (data not shown).

studies, we felt that it was appropriate, as it provided a more detailed picture of the potential usefulness and limitations associated with novel experimental drugs. In addition, the two simple metal salts, namely $Cu(ClO_4)_2{\cdot}6H_2O$ and $AgClO_4$, along with one of the most commonly used anti-cancer agents cisplatin, were included in our test protocol. It was intended that this strategy would allow us to identify whether the effects observed were due to the whole metal–phendione complex rather than the simple aquated metal ions. Additionally, it facilitates calculation of the relative potency of the phendione derivatives to that of cisplatin. Initial viability studies showed that, following 96 h exposure to the test reagents, phendione and the two metal–phendione complexes, particularly $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$, produced $IC_{50}$ values significantly lower than cisplatin. Also, these agents with the exception of $Cu(ClO_4)_2{\cdot}6H_2O$, were toxic to all four of the model cells lines. However, it is noteworthy that this effect was also observed for cisplatin, when using the two renal cell lines. Interestingly, $IC_{50}$ values for phendione and its complexes were lower in the CHANG and Hep-G2 cell lines, than in A-498 and HK-2 cells, suggesting that, in general, cells of hepatic origin may be more sensitive than renal. Therefore, it may be possible that our test agents do display a degree of selectivity for one cancer cell type over another. So, while cyto-

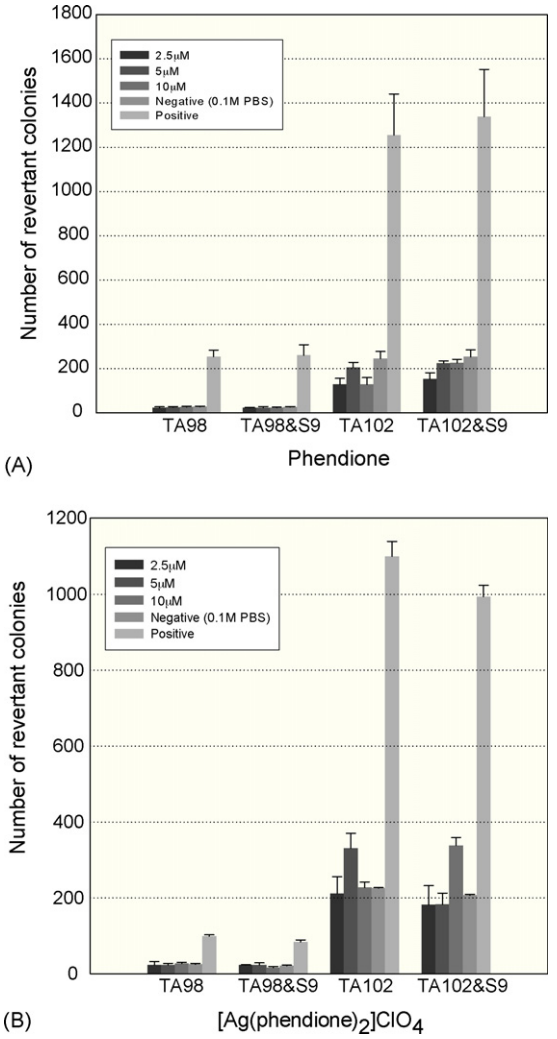



Fig. 7. Standard Ames tests were used to determine the mutagenic potential of all test compounds using *Salmonella typimurium* tester strains, TA98 and TA102, both in the presence and absence of a S9 fraction isolated from rat hepatocytes, where the animal had previously been exposed to Aroclor 1254. Results presented for (A) phendione and (B) $[Ag(phendione)_2]ClO_4$ (0–10 μM/plate) indicate that neither of these compounds are mutagenic as they do not cause a dose-dependant increase in the number of revertant colonies. Similar results were obtained for $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ (data not shown). Bars indicate ±S.E.M., $n = 3$.

selectivity is a highly desirable characteristic for all anti-cancer agents, it is often difficult to achieve, even with cisplatin, a drug currently in clinical use. Additionally, it may be possible that agents that act as potent anti-proliferative agents may display limited selectivity, as they function on a key cellular event, central to the survival of all cells. Therefore, it is possible that phendione and its metal complexes may have value in the clinical setting in the longer term, as the dose and frequency of

use could be tailored by the clinician in response to the patient's needs.

In this study, we have shown that phendione and its metal complexes display a greater degree of cytotoxicity than cisplatin. This result, although encouraging, was not unexpected, as Zhang et al. [3] had previously published findings suggesting that a ternary copper–phen complex [Cu(phen)(L-threonine)($H_2O$)]$ClO_4$, when exposed to human epithelial cell lines, displayed $IC_{50}$ values in the μM range, and with greater cytotoxicity than that observed for cisplatin. Additionally, Igdaloff et al. [38] have shown that metal-free phendione and its isomer 1,7-phenanthroline-5,6-dione could inhibit the growth of the two mouse-derived lymphoma cell lines S49 and S110. The $IC_{50}$ values recorded for phendione were 0.04–0.5 μM, and were lower than that obtained for the 1,7-isomer. These researchers concluded that phendione was the most active and postulated that the cytotoxicity observed for this ligand may be due to inhibition of DNA and RNA synthesis. However, this hypothesis was not confirmed experimentally.

In the present study, we have shown that metal-free phendione, [Cu(phendione)$_3$]$(ClO_4)_2 \cdot 4H_2O$, and [Ag(phendione)$_2$]$ClO_4$ exert a concentration-dependant cytotoxic response (Figs. 2 and 3). In addition, the results suggest that the addition of a metal to the phendione ligand amplified the cytotoxic potential. However, there remained the possibility that the observed cytotoxicity was due to the simple aquated Cu(II) and Ag(I) ions rather than the metal ions chelated by the phendione ligand. Therefore, in order to explore this theory more fully it was decided to include the simple salts $Cu(ClO_4)_2 \cdot 6H_2O$ and $AgClO_4$ in our test protocols. The results presented in Fig. 4 clearly indicate that although $AgClO_4$ was the most potent simple salt studied. However, its cytotoxicity was significantly less than that observed for either phendione or the two metal–phendione complexes. These findings are further supported by results obtained from a similar study carried out by Hidalago and Dominguez [39]. These researchers showed that $AgClO_4$ could kill human dermal fibroblasts at concentrations of 4–82 μM. So, while $AgClO_4$ processes cytotoxic properties, it would appear that it is less cytotoxic than phendione and the Cu(II) and Ag(I) phendione complexes.

It is widely recognised that any chemotherapeutic agent which can significantly reduce DNA synthesis is likely to be of particular value in controlling cancer cell division. DNA has been shown to be a key cellular target in mediating the cytotoxicity of cisplatin. Gosh et al. [23] have shown that cobalt(III) complexes of phen and phendione could intercalate with DNA, and yet retain its overall structure. However, their usefulness as anti-cancer agents remains to be defined. In this study we have attempted to determine whether interaction with DNA plays a similarly important role in mediating the cytotoxicity of phendione and its metal complexes, by probing their effect on DNA synthesis and also their ability to intercalate with it. Data presented here (Fig. 5) suggest that phendione, [Cu(phendione)$_3$]$(ClO_4)_2 \cdot 4H_2O$ and [Ag(phendione)$_2$]$ClO_4$ are capable of inhibiting DNA synthesis in both neoplastic-derived cell lines, and in a concentration-dependant manner. Furthermore, the phendione ligand appeared to be the most activity, displaying the greatest inhibition. This suggests that the phendione ligand itself is capable of playing a significant role in mediating DNA synthesis. Additionally, the current data did not explain the relationship between exposure to phendione-based compounds, decreased DNA synthesis and ultimately cell death. The possibility that these complexes, like cisplatin, could intercalate DNA and thus lead to decreased viability was explored using gel mobility shift assays [40]. The model used here proposes that intercalating ligands must possess a planar aromatic ring structure capable of hydrophobic interactions with DNA base pairs and that, on binding of the ligand, the double helix should become extended and locally uncoiled. Local unwinding of the double helix by an intercalating species causes an increase in the size of the DNA molecule. The super-coiled form of plasmid DNA migrates further than either linear or open-circular forms and can be detected by electrophoretic migration assays [41]. DNA was exposed to phendione and the metal–phendione complexes over a range of concentrations both above and below their $IC_{50}$ values (Table 1). The results presented in Fig. 6 clearly indicate that none of these agents retarded the electrophoretic mobility of supercoiled pGEM-3Z DNA, suggesting that they did not intercalate DNA, and implying that their inhibitory effects operate through an alternative mechanism. A recent study carried out by Ghosh et al. [23] showed that cobalt complexes of phen and phendione were capable of binding covalently to the DNA double helix retaining the overall structure of the helix. Additionally, they showed that these complexes could cleave plasmid DNA upon irradiation under aerobic conditions. These researchers suggest that these complexes many have anti-cancer activity, but this remains to be confirmed experimentally.

Cross et al. [42] have shown that cisplatin was mutagenic using the Ames test, suggesting that using cisplatin could lead to an increased risk of secondary malignancies. They conclude that normal cells in patients undergoing treatment with cisplatin were at an increased risk

of genotoxicity and that this must be taken into consideration when deciding on cancer treatment with this agent. In the current study, it was decided to use the standard Ames test to determine if there was a similar genotoxic risk associated with phendione and its metal-based complexes. Additional assays were carried out in the presence of an S9 fraction derived from rat hepatocytes, thereby allowing the mutagenic potential of phase I metabolites also to be determined. Results from these assays revealed that none of compounds caused a significant increase in the number of revertant colonies, either by base pair substitution or by frame shift, suggesting that they, and their phase I metabolites, were non-mutagenic (Fig. 7). These findings clearly indicate that if phendione and the metal–phendione complexes were to be employed therapeutically, their potential usefulness is not likely to be limited by mutagenicity, unlike cisplatin.

In conclusion, the above findings suggest that phendione, $[Ag(phendione)_2]ClO_4$, and particularly $[Cu(phendione)_3](ClO_4)_2{\cdot}4H_2O$ are each capable of decreasing cancer cell viability through an inhibition of DNA synthesis, but unlike cisplatin they do not cause an increased risk of genotoxicity. However, the exact mechanisms underlying the cytotoxicity of these compounds remain to be elucidated. Additional studies are currently underway in our laboratory to investigate more fully the mode of cell death induced as a result of drug exposure, along with the biochemical processes by which this is controlled. It is anticipated that the results from these studies will allow identification of key molecular targets by which the balance between cancer cell viability and death can be tipped in favour of patient survival. In addition, it is intended that these studies will facilitate the development of highly effective anti-cancer therapies, which with careful management could be tailored by the clinician in response to the patient's needs.

## Acknowledgements

This research was supported by the Technological Sector Research Programme, Strand III (2002–2005), under the European Social Fund. The research was carried out by the Centre for Pharmaceutical Research and Development (CPRD) jointly located at Institutes of Technology, Tallaght and Dublin, and the National University of Ireland, Maynooth, Co. Kildare, Ireland. The authors express their gratitude to Dr. Brian A. Murray, for his editorial support in the preparation of this manuscript. Additionally, we acknowledge Dr. Paul Tomkins, Institute of Technology, Athlone, Co. Westmeath, Ireland, for generously supplying the *Salmonella typimurium* tester strains (TA98 and TA102).

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