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In vitro cancer chemotherapeutic activity of 1,10-phenanthroline (phen), $[Ag_2(phen)_3(mal)] \cdot 2H_2O$, $[Cu(phen)_2(mal)] \cdot 2H_2O$ and $[Mn(phen)_2(mal)] \cdot 2H_2O$ ($malH_2$ = malonic acid) using human cancer cells

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Abstract

The chemotherapeutic potential of 1,10-phenanthroline (phen), and three of its transition metal 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) was determined using two human carcinoma cell lines (A-498 and Hep-G2). Phen and the three metal–phen complexes induced a concentration-dependent cytotoxic effect, with metal complexes demonstrating the greatest cytotoxic response. In comparative studies, IC_{50} values show cytotoxicity of between 3 and 18 times greater than that observed for the metal-based anti-cancer agent, cisplatin. All of the phen-based complexes inhibited DNA synthesis which did not appear to be mediated through intercalation. Also, the potential cancer chemotherapeutic application of these compounds was seen to be enhanced by results obtained from Ames tests, which showed all of the test agents and their phase I metabolites were non-mutagenic. Taken together, these results suggest that phen and the three metal–phen complexes may have a therapeutic role to play in the successful treatment and management of cancer.

Keywords: 1,10-Phenanthroline; Metal complexes; Cell carcinoma; DNA inhibition; Chemotherapeutic potential

1. Introduction

Recently there have been a number of reports highlighting the use of transition metal complexes as anticancer agents [1]. Probably the best known of these is cisplatin [*cis*-diamminedichloroplatinum(II)]. It has been widely used in the treatment

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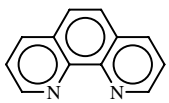
of a variety of cancers, especially testicular cancer, with a 70–90% cure rate. When combined with other drugs, it has successfully been used to treat brain, ovarian, bladder and breast cancer [2]. The clinical success of cisplatin is limited by its significant side effects, such as nausea, vomiting and severe nephrotoxicity [2]. The use of cisplatin and related platinum complexes as anticancer agents has stimulated a search for other active transition metal complexes which are as effective, but with lesser side effects.

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 [3]. Furthermore, when metal-free *N*,*N*'-chelating bases are found to be bioactive it is usually assumed that sequestering of trace metals in situ was involved, and that the resulting metal complexes were the active species [4,5]. Previous work has shown that the 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 the growth of *Candida albicans* by around 95% in a concentration range of 1.25–5.0 μg/ml [6,7]. These complexes induced extensive changes in the internal structure of cells, including retraction of the cytoplasm, nuclear fragmentation and disruption of the mitochondria [8]. From mechanistic studies it was established that both metal-free phen and metal–phen complexes affected mitochondrial function, retarded synthesis of cytochromes *b* and *c*, and uncoupled cellular respiration. Treatment of fungal cells with Cu(II) and Ag(I) complexes resulted in a reduced amount of ergosterol in the cell membrane with consequent increases in permeability. Cells exposed to metal-free phen and its Cu(II) and Mn(II) complexes [but not the Ag(I) complex] demonstrated an elevation in oxygen uptake. The general conclusion was that these drugs damaged mitochondrial function and uncoupled respiration. Furthermore, the fact that they were not uniformly active suggested that their bioactivity was dependent on the central metal ion in the complex.



1,10-phenanthroline
(phen)

Fig. 1. Structure of 1,10-phenanthroline (phen).

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 stimulation of reactive oxygen species. Transition metal cations, such as Cu(II) and Fe(II), can bind to negatively charged DNA and have been shown to play an important role in the local formation of ·OH radicals [9,10]. One of the consequences of high copper levels in the body is an increase in the rate of radical formation leading to oxidative damage [11]. 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 [11]. Another possible consequence is apoptosis. Tsang et al. [12] 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. [13] 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 [14].

The primary aim of the current study was to determine the cancer chemotherapeutic potential of metal-free phen, $[Cu(phen)_2(mal)]\cdot 2H_2O$, $[Mn(phen)_2(mal)]\cdot 2H_2O$ and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$, using two human-derived cancer model cell lines of hepatic (Hep-G2) and renal (A-498) origin. In order to illustrate that the effect observed was due to the complexes rather than the free metal ions, the anti-tumour activities of the simple Cu(II), Mn(II) and Ag(I) salts, $Cu(ClO_4)_2$, $Mn(ClO_4)_2$ 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. Furthermore, aspects of the molecular mechanisms underlying the cytotoxic response were probed by investigating the role of the complexes in mediating DNA synthesis with intercalation. This work represents the first thorough assessment of the potential application of phen,

$[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ as novel therapeutic agents for the treatment of cancer.

2. Materials and methods

2.1. Test compounds

1,10-Phenanthroline (phen), cisplatin, $Cu(ClO_4)_2 \cdot 6H_2O$, $Mn(ClO_4)_2 \cdot 6H_2O$, $AgClO_4$, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, Ireland Ltd., whilst $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ were synthesised and characterised by the methods outlined by McCann et al. [6]. All cell culture reagents and media were purchased from Euroclone, UK, unless otherwise stated.

2.2. Model cell lines

A-498 (human kidney adenocarcinoma) and Hep-G2 (human hepatocellular carcinoma) cells were purchased from the American Type Culture Collection, Manassas, USA. Both cell types 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). Both 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

Phen, $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$, $[Ag_2(phen)_3(mal)] \cdot 2H_2O$, $Cu(ClO_4)_2 \cdot 6H_2O$, $Mn(ClO_4)_2 \cdot 6H_2O$, $AgClO_4$ and cisplatin were dissolved in DMSO, diluted in culture media and used to treat model cell lines over a drug concentration range of 0–1000 μM 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 5×10^4 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 according to the method described by Mosmann [15]. 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 phen and each of the three phen–metal complexes on DNA synthesis was determined using a 5-bromo-2-deoxyuridine (BrdU) colourimetric incorporation assay [16]. One hundred microliters of A-498 and Hep-G_2 cells were seeded at a density of 5×10^4 cells/well into 96-well plates and allowed to adhere overnight at 37 °C in a humid atmosphere with 95% air and 5% CO_2. Cells were incubated with test drug for 96 h. BrdU was added to each well to give a final concentration of 10 μM and incubated for 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 the 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, and were protected from light. The reaction was then stopped by the addition of 1 M H_2SO_4 (25 μl/well). 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 the 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 A_{260}/A_{280} 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 phen, metal–phen complexes and three simple metal salts, according to the method described by Lorcozio and Long [17]. 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; and 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 phen and three metal–phen complexes at concentration points close to the IC_{50} value (0–10 μM) as determined by MTT assay (Section 2.3). *Salmonella typhimurium* tester strains, TA 98 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 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 [18].

3. Results

All test agents were incubated with the two model cell lines for a period of 96 h, after which cellular viability was determined using MTT. Results obtained for phen, its three metal–phen complexes and simple metal salts were determined and are presented in Figs. 2 and 3. These graphs were used to calculate the IC_{50} values and are presented in Table 1. Phen and the three metal–phen complexes displayed a concentration-dependent cytotoxic profile in both cell lines. Since the IC_{50} values for $[Cu(phen)_2(mal)]\cdot 2H_2O$, $[Mn(phen)_2(mal)]\cdot 2H_2O$ and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ were statistically slower than that for metal-free phen in A-498 cells, it suggested that coordinated metal ions play a major role in mediating potency of the complex. Also, while the IC_{50} values for metal-free phen and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ on Hep-G2 cells were not statistically different, $[Cu(phen)_2(mal)]\cdot 2H_2O$ and $[Mn(phen)_2(mal)]\cdot 2H_2O$ displayed a greater effect against this cell line. This latter result suggests that in general, Hep-G2 may be more sensitive than A-498 cells. Significantly, phen and its metal complexes exhibited substantially greater cytotoxicity than cisplatin and this was found in both model cell lines.

In order to prove that the cytotoxicity observed was due to the metal–phen complexes, rather than to simple aquated metal ions of the type $[M(H_2O)_x]^{y+}$ [M = Cu(II), Mn(II), Ag(I)], the phen-free metal salts $Cu(ClO_4)_2\cdot 6H_2O$, $Mn(ClO_4)_2\cdot 6H_2O$ and $AgClO_4$ were screened against both model cell lines (Fig. 3, Table 1). The data suggest that only $AgClO_4$ displayed notable activity, being more potent against Hep-G2 cells than cisplatin, but less effective than phen and its metal complexes.

Table 1
Cytotoxic potential of phen and its metal-based complexes was determined using A-498 and Hep-G2 cells, following 96 h continuous incubation in the range of 0–1000 μM, using MTT viability assay

Compound	A-498 IC_{50} (μM) ± SEM	Hep-G_2 IC_{50} (μM) ± SEM
Phen	5.8 ± 0.31	4.1 ± 0.54
$[Cu(phen)_2(mal)]\cdot 2H_2O$	3.8 ± 0.41[a]	0.8 ± 0.02[a]
$[Mn(phen)_2(mal)]\cdot 2H_2O$	4.2 ± 0.57[a]	0.8 ± 0.07[a]
$[Ag_2(phen)_3(mal)]\cdot 2H_2O$	4.0 ± 0.32[a]	4.7 ± 0.26
$Cu(ClO_4)_2\cdot 6H_2O$	973.3 ± 26.67	>1000.00
$Mn(ClO_4)_2\cdot 6H_2O$	880.0 ± 20.00	626.7 ± 27.29
$AgClO_4$	44.4 ± 2.34	7.6 ± 0.70
Cisplatin	14.0 ± 1.00	15.0 ± 2.65

A graph of viability versus drug concentration was used to calculate all IC_{50}values (μM), $n = 5$.

[a] Value is statistically distinct from that of the parent ligand at $p < 0.05$.

In an attempt to elucidate the events responsible for the reduction in cellular viability, the effect on DNA synthesis was determined using BrdU incorporation assays. Results obtained suggest that phen and the metal–phen complexes caused a dose-dependent decrease in DNA synthesis, following a 96 h incubation period (Fig. 4). This trend is consistent with viability data obtained from MTT assays (Fig. 2).

To explore the relationship between the observed cytotoxic response and the possibility that DNA was a molecular target, intercalation studies were carried out using electrophoretic mobility shift assays. Additionally, the planer structure of the phen ligand (Fig. 1) might imply the possible involvement of intercalation in mediating the toxic response. Treatment of pGEM-3Z plasmid DNA with phen, the metal–phen complexes and the simple metal salts at concentrations of 1, 10 and 200 μM did not alter the migration of any of the three forms of DNA (super-coiled, linear, and open-circular), unlike the positive control doxorubicin (Fig. 5). This data indicates that phen and its metal-based complexes do not intercalate DNA and so may function by an alternative mechanism.

Additionally, in order to determine the mutagenic potential of these compounds, the Standard Ames test was carried out. The results presented in Fig. 6 showed that phen, $[Cu(phen)_2(mal)]\cdot 2H_2O$, and their phase I metabolites were non-mutagenic. Similar results were obtained for $[Mn(phen)_2(mal)]\cdot 2H_2O$ and $[Ag_2(phen)_3(mal)]\cdot 2H_2O$ (data not shown). Finally, the results presented here confirm that phen, and particularly the

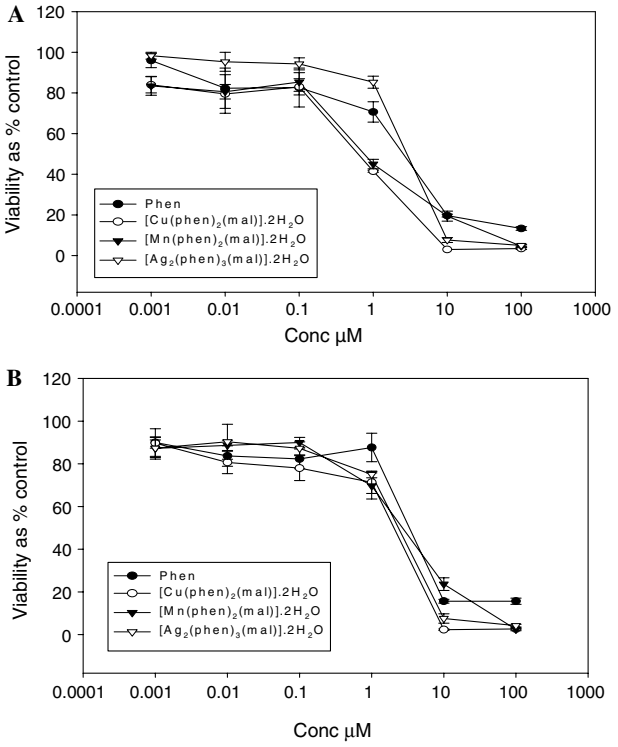


Fig. 2. Effects of phen and the three metal-based phen complexes on the viability of (A) A-498 (B) Hep-G_2 cells. All compounds showed a concentration-dependent decrease in viability and in both cell lines, following 96 h incubation (0–1000 μM). Results are expressed as percentage viability of solvent-treated control cells. Bars indicate ± SEM, $n = 3$.

metal–phen complexes, are potent cytotoxic agents, capable of decreasing both cellular viability and DNA synthesis, but without the involvement of intercalation or mutation.

4. Discussion

Phen and its copper complexes have previously been shown to exert a range of biological activities, such as anti-tumour [19], anti-*Candida* [20], anti-mycobacterial [21] and anti-microbial [22] effects. In this study we attempted to identify the chemotherapeutic potential of phen and the three metal–phen complexes, $[Cu(phen)_2(mal)] \cdot 2H_2O$, $[Mn(phen)_2(mal)] \cdot 2H_2O$, and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$, along with characterising key aspects of their in vitro mode of action. Additionally, the current study represents the first thorough examination of this potential application of phen and metal-based phen complexes as novel therapeutic agents for the treatment of cancer. Two human-derived epithelial cell lines were used throughout. Initial studies were carried out to determine IC_{50} values for phen, the three metal–phen complexes, along with the three simple metal salts and cisplatin. It was intended that this approach would allow us to identify whether the effects observed were due to the whole metal–phen complex rather than the simple aquated metal ion, along with comparing their relative potency to that of cisplatin. Initial viability studies show that following 96 h exposure, phen and each of the metal–phen complexes had IC_{50} values significantly lower than that recorded for cisplatin. This result, although encouraging, was not unexpected, as Zhang and Lippard [23] had previously published findings to suggest 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 cytotoxicity greater than cisplatin. Findings from each of these earlier studies serve to substantiate the results obtained here. Additionally, the present study has shown that metal-free phen and the metal–phen complexes displayed a concentration-dependent cytotoxic response, with Hep-G2 cells appearing to be the most susceptible (Table 1, Fig. 2). Also, with the exception of $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ in Hep-G2 cells, the results suggest that the addition of a

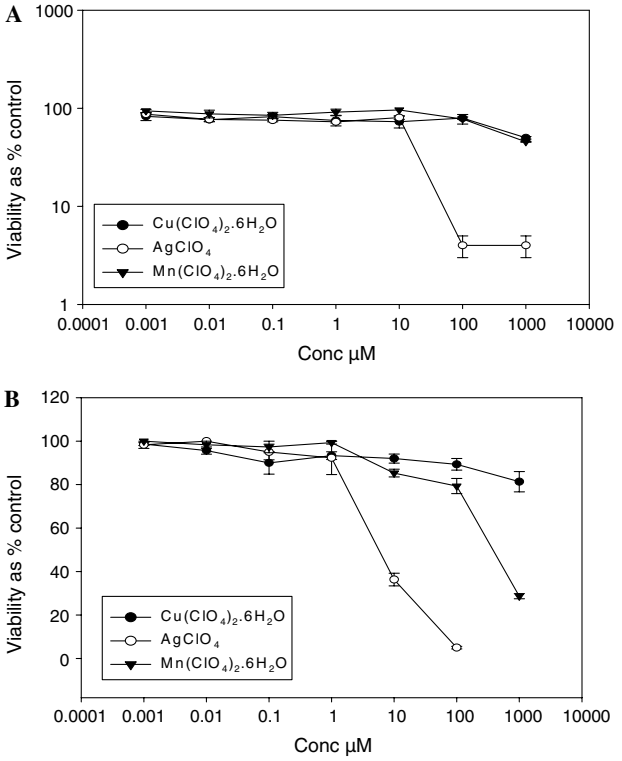


Fig. 3. Effects of simple transition metal salts, $Cu(ClO_4)_2 \cdot 6H_2O$, $Mn(ClO_4)_2 \cdot 6H_2O$ and $AgClO_4$ on the viability of (A) A-498 and (B) Hep-G_2 cells. All salts promoted a decrease in viability following 96 h incubation (0–1000 μM). However, $AgClO_4$ appeared to be the most potent, and in both cell lines. Results are expressed as percentage viability of solvent-treated control cells. Bars indicate ± SEM, $n = 3$.

metal to the phen ligand served to enhance cytotoxicity. The possibility that observed cytotoxicity for the metal–phen complexes may be due to the phen-free simple solvated Cu(II), Mn(II) and Ag(I) ions was also tested. The results obtained clearly indicated that although $AgClO_4$ was the most potent simple salt (Fig. 3) it was still less cytotoxic than phen and the metal–phen complexes. Similar results have previously been published where $AgClO_4$, at concentrations in the range of 4–82 μM were shown to kill human dermal fibroblasts [24]. So, while $AgClO_4$ processes cytotoxic properties, it would appear that it is less cytotoxic than either phen or the three metal–phen complexes studied here.

In an attempt to explore the molecular mechanism(s) underlying the observed cytotoxic response, BrdU incorporation studies were conducted. DNA synthesis is known to be critical to cell division. Therefore, any chemotherapeutic agent that can significantly reduce DNA synthesis is likely to be of particular interest in controlling cancer cell division. The data presented here suggest that phen and its metal-based complexes are capable of inhibiting DNA synthesis in both cell lines and in a concentration-dependent manner. However, again Hep-G2 cells appeared to be most sensitive. Furthermore, the presence of a metal ion coordinated to the phen ligand did not serve to enhance the observed inhibition. This suggests that the phen ligand itself may play a significant role in mediating DNA inhibition. However, the current data did not explain the relationship between exposure to phen-based compounds and decreased DNA synthesis and ultimately cell death, suggesting the necessity for additional mechanistic studies.

A number of researchers have focused considerable attention on determining whether DNA could be a primary target for phen-containing compounds. Studies have shown that bis(1,10-phenanthroline)-copper(II) can efficiently cleave DNA in the presence of thiol and hydrogen peroxide [25–27]. These researchers have suggested that DNA strand scission may occur through non-covalently binding to the minor groove of DNA and reacting with hydrogen peroxide. In 2004, Zhang et al. [28] showed that a ternary copper(II) complex of phenanthroline and L-threonine were capable of both

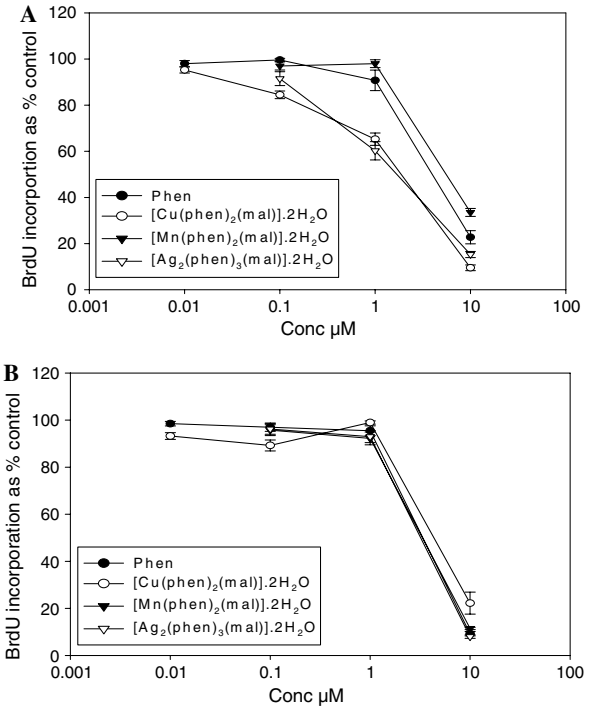


Fig. 4. Effects of phen and three metal-based phen complexes on DNA synthesis in (A) A-498 and (B) Hep-G2 cells, following continuous exposure for 96 h. All compounds produced a dose-dependent decrease in DNA synthesis. Bars indicate ± SEM, $n = 3$.

binding to DNA by intercalation and were also able to cleave DNA in the presence of ascorbate. However, they suggest that the introduction of the L-threonine rather than the phen ligand was primarily responsible for these effects. Additionally, Meheswari and Palaniandavar [29] used a number of tetrammine ruthenium(II) complexes of modified phen to show that the planarity of the modified phen ligand plays an important role in dictating DNA binding affinity. More recently, Heffeter et al. [30] found that a lanthanum derivative of phen, [tris(1,10-phen)lanthanum(III) trithiocyanate, was a potent anticancer agent and this suggest that DNA was not likely to be the primary target. This theory was based on a lack of evidence for any significant interaction with or damage to DNA. In the current study we attempted to explore the possibility that phen and the three metal–phen complexes could intercalate DNA. The model used to demonstrate intercalation was gel mobility shift assays originally advanced by Waring [31]. This model 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 results in an increase in the size of the DNA molecule. The super-coiled form of plasmid DNA migrates further than linear or open circular forms and can be detected by electrophoretic migration assays [32]. DNA was exposed to phen and the metal–phen complexes over a range of concentrations above and below their IC_{50} values (Table 1). The results presented in Fig. 5 clearly indicate that none of these agents were capable of retarding the electrophoretic mobility of supercoiled

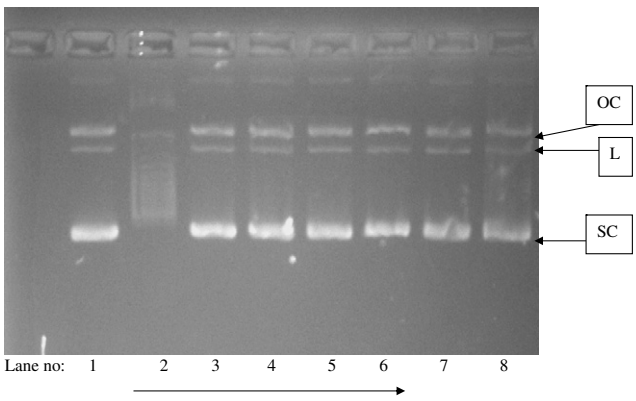


Fig. 5. Electrophoretic mobility shift assays were used to determine the effects of phen and $[Cu(phen)_2(mal)] \cdot 2H_2O$ on the migration of pGEM-3Z supercoiled plasmid DNA for 2 h at 37 °C, analysed by agarose electrophoresis and stained with ethidium bromide. Results indicate that neither compound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

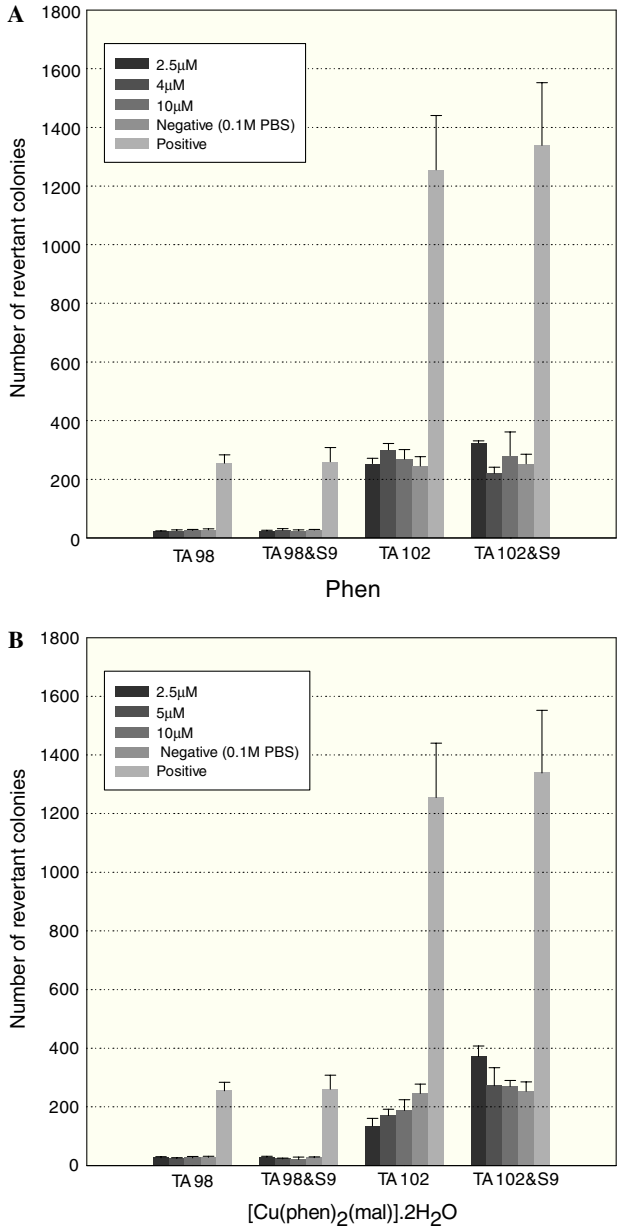


Fig. 6. Standard Ames tests were used to determine the mutagenic potential of all test compounds using *Salmonella typhimurium* tester strains, TA 98 and TA102, both in the presence and absence of S9 fraction isolated from rat hepatocytes, where the animal had previously been exposed to Aroclor 1254. Results are presented for phen and $[Cu(phen)_2(mal)] \cdot 2H_2O$ (0–10 μM/plate) and indicate that neither of these compounds are mutagenic as they do not cause a dose-dependent increase in the number of revertant colonies. Similar data were obtained for $[Mn(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ (data not shown). Bars indicate ± SEM, $n = 3$.

pGEM-3Z DNA, suggesting that they did not intercalate DNA. These findings imply that the inhibition observed may operate through an alternative mechanism. In addition, they also serve to confirm the findings of Heffeter et al. [30].

Additionally, it was decided to determine the mutagenic potential of each of the compounds using the standard Ames test. Further assays were also carried out in the presence of an S9 fraction derived from rat hepatocytes, thereby allowing the mutagenic potential of phase I metabolites to be determined. The results presented in Fig. 6 show that none of the 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 metabolites were non-mutagenic. These findings clearly indicate that if phen and metal–phen complexes were to be employed therapeutically, their potential is not likely to be limited by mutagenicity.

In conclusion, the above findings suggest that phen, $[Mn(phen)_2(mal)] \cdot 2H_2O$, and particularly $[Cu(phen)_2(mal)] \cdot 2H_2O$ and $[Ag_2(phen)_3(mal)] \cdot 2H_2O$ are capable of decreasing cancer cell viability through an inhibition of DNA synthesis. Studies are currently underway in our laboratory to investigate more fully the mechanisms by which phen and the three metal–phen complexes control cancer cell viability. It is intended that the results from these studies will allow identification of key molecular targets, and in doing so will assist in elucidating their mechanisms of action, along with facilitating the development of highly effective anti- cancer therapies.

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