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Molecular and cellular pharmacology

Anti-cancer activity and mutagenic potential of novel copper(II) quinolinone Schiff base complexes in hepatocarcinoma cells

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ABSTRACT

This study determined the cytotoxic, cyto-selective and mutagenic potential of novel quinolinone Schiff base ligands and their corresponding copper(II) complexes in human-derived hepatic carcinoma cells (Hep-G2) and non-malignant human-derived hepatic cells (Chang). Results indicated that complexation of quinolinone Schiff bases with copper served to significantly enhance cytotoxicity. Here, the complex of (7E)-7-(3-ethoxy-2-hydroxybenzylideamino)-4-methylquinolin-2(1H)-one (TV117-FM) exhibited the lowest IC50 value (17.9 μM) following 96 h continuous exposure, which was comparable to cisplatin (15.0 μM). However, results revealed that TV117-FM lacked cytoselectivity over non-malignant cells. Additionally, the complex was minimally effluxed from cells via Pglycoprotein (P-gp) and was shown to be non-mutagenic in the Standard Ames test. Furthermore, BrdU incorporation assays showed that it was capable of inhibiting DNA synthesis in a concentrationand time-dependent manner. However, inhibition was not as a consequence of DNA intercalation, as illustrated in electrophoretic mobility shift assays. Interestingly, it was shown that the ligand was capable of inhibiting the action of topoisomerase II, but this was lost following complexation. This indicated that the mechanism of action of the novel copper(II) complex was different from that of the parent ligand and suggests that TV117-FM may have a therapeutic role to play in the treatment of hepatocellular carcinoma. Studies are currently underway to elucidate the exact in vitro mechanism of action of this novel, metal-based anti-cancer agent.

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1. Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide with particularly high incidences in East Asia and sub-Saharan Africa (Venook et al., 2010). In Ireland, 180 people are diagnosed annually with HCC, (Irish Cancer Society, 2011). HCC is considered to be a highly malignant disease, with an extremely poor prognosis, and chronic infections with both hepatitis B and C viruses (HBV and HCV) are known to be important risk factors in its development. It has also been linked to initial chronic hepatitis without direct liver cirrhosis. In addition, researchers have suggested that HCV, aflatoxin, alcohol and non-alcoholic fatty liver disease (NAFLD) may either act independently or interact with HBV to induce liver cirrhosis (El-Serag and Rudolph, 2007). Also, generation of reactive oxygen species (ROS) via mitochondrial dysfunction, coupled with down-

regulation of oxido-reductive enzymes have also been linked to hepatocarcinogenesis (Marra et al., 2011). Moreover, it has been shown that patients' origin along with aetiological factors of HCC development may impact on the type of mutations encountered in this disease. Colombino et al. (2012) reported a correlation between mutational activation of the BRAF and PIK3CA genes, and the tumourigenesis of HCC, in Southern Italy. These genes play key roles in both cellular proliferation and survival. Therefore, control of these factors may limit the progression of this disease.

Treatment of the disease is dependent upon the extent of liver dysfunction or cirrhosis. In early stage HCC, treatment may involve resection, transplantation or local ablation using trans-catheter arterial chemoembolisation (TACE), while intermediate stage HCC is generally treated with TACE and/or yttrium-90 radioembolisation. However, sub-optimal efficacy of these treat-ments remains a challenge which needs to be addressed (Wörns and Galle, 2010). Recently, the tyrosine protein kinase inhibitor, sorafenib has been approved for the treatment of advanced stage HCC (Keating and Santoro, 2009). In addition, sorafenib, adminis-tered in combination with octreotide-LAR (long-acting release), a somatostatin analogue, has been shown to be active and well

tolerated in the treatment of advanced HCC (Caraglia et al., 2011; Marra et al., 2011).

Quinolinones are a class of heterocyclic compounds known to possess diverse therapeutic activities. They act as the structural backbone of many medicinal drugs in the treatment of a range of diseases including; inflammatory diseases, psychosis and cancer (Burris et al., 2002; Kulakarni et al., 2006). However, interest in the use of quinolinones as anti-cancer agents arose from the finding that vesnarinone, a quinolinone analogue, used in the treatment of congestive heart failure, was capable of attenuating the growth of cancer cells, both in vitro and in vivo (Sato et al., 1995, 1996; Nio et al., 1997; Kawai et al., 1998). More recently, a novel class of quinoline-2-ones were shown to be potent photochemotherapeutic agents with IC_{50} values at the submicromolar level (Barraja et al., 2010, 2011). Furthermore, quinolinone derivatives have been reported to possess pro-apoptotic characteristics (Claassen et al., 2009). Additionally, Schiff base ligands and their Cu(II) complexes have shown enhanced anti-cancer activity (Zhao et al., 2007). Moreover, our research group have previously reported the in vitro anti-fungal potential of novel copper(II) complexes of coumarin-derived Schiff bases, which are structurally similar to quinolinones (Creaven et al., 2009) in addition to elucidating the anti-cancer potential of novel copper-, silver- and manganese-phenantholine (phen) and phendione complexes (Deegan et al., 2006, 2007). Also, the function of preventing de novo replication of DNA by topoisomerase II (TopoII) inhibition is a desirable characteristic of any novel chemotherapeutic agent, and coumarin-based complexes have also been shown to be potent TopoII inhibitors (Finn et al., 2004). It is hypothesised that these novel compounds and their corresponding Cu(II) complexes may act in a manner similar to that of the copper complexes of phen and phendione (Deegan et al., 2006). Presently, the anti-cancer potential of such quinolinone Schiff base copper complexes, has yet been reported in the literature.

The aim of the current study was to evaluate the in vitro cytotoxic potential of a series of novel quinolinone Schiff bases and their corresponding copper(II) complexes, using a hepatocellular carcinoma model cell line, Hep-G_2. Subsequently, the cyto-selective, mutagenic, and TopoII inhibitory potential of the most potent assessed agent(s) was determined. Where possible, comparative studies were carried out with one of the most clinically used anti-cancer agents, cisplatin. This served to highlight the clinical potential of members of this series of compounds.

2. Materials and methods

2.1. Test compounds and reagents

Cisplatin, copper perchlorate and actinomycin D were purchased from Sigma-Aldrich, Ireland. All quinolinone-derived Schiff bases and corresponding Cu(II) complexes were synthesised and purified as described by Creaven et al. (2010). Structure and purity were confirmed by thin layer chromatography, infra red analysis, ^{1}H- and ^{13}C-NMR spectroscopy, along with elemental analysis. Assessed agents were solubilised in dimethyl sulfoxide (DMSO, 0.25%, v/v). pGEM-3Z plasmid DNA was purchased from Promega and BrdU cell proliferation kits were obtained from Calbiochem, U.K. Topoisomerase II relaxation kits were purchased from Inspiralis, U.K. All other chemicals, reagents and growth media were purchased from Sigma-Aldrich, Ireland, unless otherwise stated.

2.2. Model cell lines

Hep-G_2 (human hepatocellular carcinoma) and Chang (non-neoplastic human hepatic, with possible HeLa contamination) cells were purchased from the American Type Culture Collection,

Manassas, USA. These cells were maintained in Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced Salt Solution, 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.

Chinese hamster ovary cells (CHO-K1 and CHC5) were kindly provided by Dr. V. Ling, Ontario, Canada. CHO-K1 cells were maintained in Ham's F-12 media, containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal bovine serum. CHC5 cells were maintained in EMEM, alpha modification (without ribonucleosides and deoxynucleosides), containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal bovine serum. All cell lines were grown at 37 °C in a humidified atmosphere and in the presence of 5% CO_2.

2.3. Assessment of anti-proliferative activity using MTT assay

All quinolinone-derived Schiff bases and their corresponding Cu(II) complexes were dissolved in DMSO, diluted in culture media and used to treat model cell lines over a range of drug concentrations for periods of 4, 24 and 96 h. The maximum percentage of DMSO present in any well was 0.25% (v/v). Cells were seeded into sterile 96-well flat-bottomed plates (Sarstedt) at a density of 2.5×10^4 cells/ml and grown in 5% (v/v) CO_2 at 37 °C. A miniaturised viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was carried out according to the method described by Mosmann (1983). In metabolically active cells, MTT is reduced by the mitochondrial enzyme succinate dehydrogenase to form insoluble formazan crystals that are subsequently solubilised, and the optical density (OD) measured spectrophotometrically. This method is commonly used to illustrate inhibition of cellular proliferation. Drug-treated cells were assayed by addition of 20 μl of 5 mg/ml MTT in 0.1 M phosphate buffered saline (PBS), pH 7.4. Following incubation for 4 h at 37 °C, the overlying medium was aspirated with a syringe and 100 μl of DMSO was added to dissolve the formazan crystals. Plates were agitated to ensure complete dissolution of crystals and OD was measured at 550 nm using a Tecan Sunrise microtitre plate reader. Viability was expressed as a percentage of solvent-treated control cells. This assay had five replicates per concentration point, and each experiment was carried out on at least the separate occasions. The IC_{50} was calculated for each assessed agent and defined as the concentration (μM) causing a 50% reduction in cellular proliferation.

2.4. Plate incorporation mutagenicity assay

The possible genotoxicity of assessed agents was investigated using the Standard Ames bacterial mutation assay described by Maron and Ames (1983). The primary tester strains used were Salmonella typhimurium strains TA98 and TA100 (Trinova, Germany), which detect mutation by frame-shift and base-pair substitution, respectively. Both strains carry the ampicillin resistance plasmid pKM101. Tester strains were cultured in Oxoid nutrient broth No. 2 (Fannin, Ireland) to a cell density of 1×10^9 cells/ml (OD of 0.60 at 605 nm) and maintained on minimal glucose plates, supplemented with 0.5 mM histidine/biotin and ampicillin.

To carry out the Ames test, top agar [0.3% (w/v) agar and 0.5% (w/v) NaCl] was supplemented with 10 ml of 0.5 mM histidine/biotin per 100 ml of agar, immediately prior to use. A 2 ml aliquot of top agar was distributed into a sterile universal tube. A mammalian enzyme activation system was also included, which consisted of the S9 liver fraction of a male Sprague–Dawley rat, induced with aroclor 1254. A 500 μl aliquot of S9 mixture (10%, v/v), 100 μl of assessed agent at the appropriate concentration dissolved in 0.1 M PBS, pH 7.4, and 100 μl of tester strain were added to 2 ml

aliquots of molten top agar, mixed and quickly poured over the surface of minimal glucose plates lacking histidine/biotin. All plates were then allowed to solidify, inverted and then incubated and protected from light, at 37 °C for 72 h. Following incubation, the number of revertant colonies per plate was determined. Each assessed agent concentration was analysed in triplicate, both in the presence and absence of the S9 enzyme activation system. Negative control plates used consisted of tester strain and S9 mix alone, while the solvent-treated control consisted of tester strain, S9 mixture and solvent vehicle (no assessed agent). This approach allowed identification and quantification of spontaneous bacterial revertants. Positive controls included the known mutagens, 4-nitro-quinoline-*N*-oxide (NQNO) for TA98 (1–1000 ng/plate) and sodium azide (0.5–10 µg/plate) for TA100. The metabolising activity of the rat liver S9 was tested in both strains using 2-aminoanthacene, a mutagen requiring metabolic activation in order to induce mutation.

2.5. DNA binding studies

The ability of the compounds to bind to DNA by intercalation was investigated using the electrophoretic mobility shift assay described by Lorico and Long (1993). Drug binding reactions were carried out in a final volume of 10 µl containing 5 µl of 50 mM Tris–HCl, pH 7.5 or assessed agent diluted in 50 mM Tris–HCl, pH 7.5 at two-fold concentration and 5 µl pGEM-3Z plasmid DNA (Promega) at a concentration of 0.05 µg/µl. Reactions were carried out at 37 °C for 2 h and then terminated by the addition of 2.5 µl of termination buffer [50% (v/v) glycerol; 20 mM EDTA; 0.25% (w/v) bromophenol blue]. Reactions were immediately electrophoresed on a 1% (w/v) agarose gel containing ethidium bromide (5 µg/ml) in TAE [40 mM Tris-acetate pH 8.5; 1 mM EDTA]. DNA bands were visualised by irradiation at 300 nm and photographed using a Syngene G-Box imaging system. Doxorubicin (25 µM) was used as positive control, while the negative control consisted of pGEM-3Z plasmid DNA incubated with 50 mM Tris–HCl, pH 7.5.

2.6. DNA synthesis studies

The effect of assessed agent on DNA synthesis was determined using the 5-bromo-2-deoxyuridine (BrdU) colourimetric incorporation assay (Calbiochem, U.K.). This assay is based on the incorporation of the halogenated pyrimidine analogue BrdU, into the DNA of proliferating cells and represents a non-radioactive alternative to the [³H]-thymidine incorporation assay (Portsmann et al., 1985). BrdU incorporated into cellular DNA was detected by immunoassay. Cell lines were seeded at a density of 2.5×10^4 cells/ml into a 96 well plate in a final volume of 100 µl/well and allowed to adhere overnight at 37 °C in a humid atmosphere, with 95% air and 5% CO_2. Assessed agent was prepared as described in Section The test compounds and reagents. A 100 µl aliquot of each assessed agent concentration was added to 3 replicate wells. The experimental controls consisted of two negative controls; (i) vehicle-treated cells and (ii) untreated control cells and the positive control (iii) actinomycin-D treated cells. Plates were incubated with assessed agent for 4, 24, and 96 h at 37 °C in a humid atmosphere with 95% air and 5% CO_2. BrdU label (10 µM) was added to each well and the plate incubated for a further 4 h at 37 °C. Incorporation was quantified using ELISA (Calbiochem, U.K.).

2.7. DNA topoisomerase II strand-passing assay

pGEM-3Z plasmid DNA was used to measure the strand-passing ability of topoisomerase II in the presence of assessed agent. Reactions were carried out in a final volume of 20 µl. These consisted of the following; 2 µl of topoisomerase II buffer [100 mM Tris–HCl; pH 7.9; 500 mM NaCl; 500 mM KCl; 500 mM MgCl₂; 1 mM EDTA; 150 µg/ml BSA and 10 mM ATP], 3 µl of pGEM-3Z plasmid DNA (0.1 µg/µl), 2 units of topoisomerase II [(1 unit/µl) in 10 mM sodium phosphate, pH 7.1] containing; [(50 mM NaCl, 0.2 mM DTT, 0.1 mM EDTA, 0.5 mg/ml BSA and 10% (v/v) glycerol] (Inspiralis, UK), 3 µl deionised H₂O and 10 µl of assessed agent in water at a two-fold excess concentration. Reactions were mixed gently, incubated at 30 °C for 30 min and protected from light. Topoisomerase reactions were terminated by the addition of 3 µl of 7 mM EDTA, containing 0.77% (w/v) SDS. Experimental controls consisted of pGEM-3Z plasmid DNA alone, topoisomerase II and pGEM-3Z plasmid DNA, topoisomerase II, pGEM-3Z plasmid DNA and the topoisomerase II inhibitor, novobiocin (10 µM) (Sigma). Finally, 4.6 µl of 6 X tracking dye [0.05% (w/v) bromophenol blue and 50% (v/v) glycerol] was added to each reaction tube. This was applied to 1% (w/v) agarose gels and electrophoresed in TAE buffer [40 mM Tris-acetate pH 8.5; 1 mM EDTA] for 2.5 h at 80 V. Gels were stained in ethidium bromide (5 µg/ml), destained in deionised H₂O, visualised and photographed by irradiation at 300 nm using a Syngene G-Box imaging system.

2.8. Statistical analyses

Statistical analyses were performed using the software package SigmaStat™ 8.0. Statistical analysis of solvent-treated cells compared with cells treated with test agent was evaluated using one-way ANOVA (Analysis Of Variance). A probability of 0.05 or less was deemed statistically significant. The standard error of the mean (S.E.M.) was estimated for all values. The following notation was used throughout; $*P < 0.05$, $**P < 0.01$.

3. Results

3.1. Anti-proliferative and cyto-selective properties

The dose-dependent anti-proliferative effect of quinolinone Schiff bases and their copper complexes was assessed using Hep-G₂ cells and the MTT bioassay. The MTT assay was carried out as described in Section The assessment of anti-proliferative activity
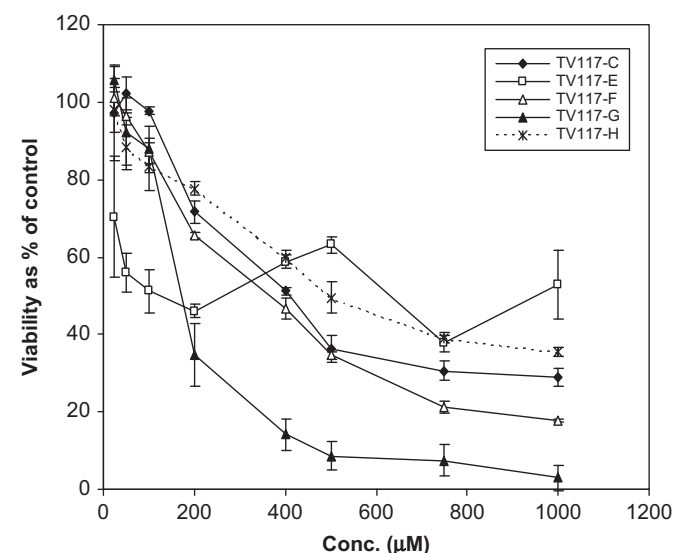
Fig. 1. Anti-proliferative effect of quinolinone Schiff bases on Hep-G₂ cells, as assessed by MTT. Results presented represent anti-proliferative assays carried out in 96 well plates with continuous exposure to assessed agent (0–1000 µM) for 96 h. All assessed agents except TV117-E caused a dose-dependent decrease in cellular proliferation. Results obtained were expressed as a percentage of solvent-treated control cells. Bars indicate mean ± S.E.M., *n*=3.

using MTT assay. Cells were continuously exposed to assessed agent for 96 h, and their effect on cellular proliferation determined. Dose-response curves of cell viability versus assessed agent concentration were used to calculate the IC_{50} value for each assessed agent (Figs. 1 and 2, Table 1).

Results obtained indicated that all assessed agents, with the exception of copper perchlorate and TV117-E, caused a concentration-dependent decrease in cellular proliferation in Hep-G_2 cells (Fig. 1). The most potent quinolinone Schiff base observed was TV117-G, with an IC_{50} value of 175.7 μM. When quinolinone Schiff bases were complexed with copper, the anti-cancer potential of all compounds was significantly increased (Fig. 2 and Table 1). Interestingly, TV117-F possessed an IC_{50} value of
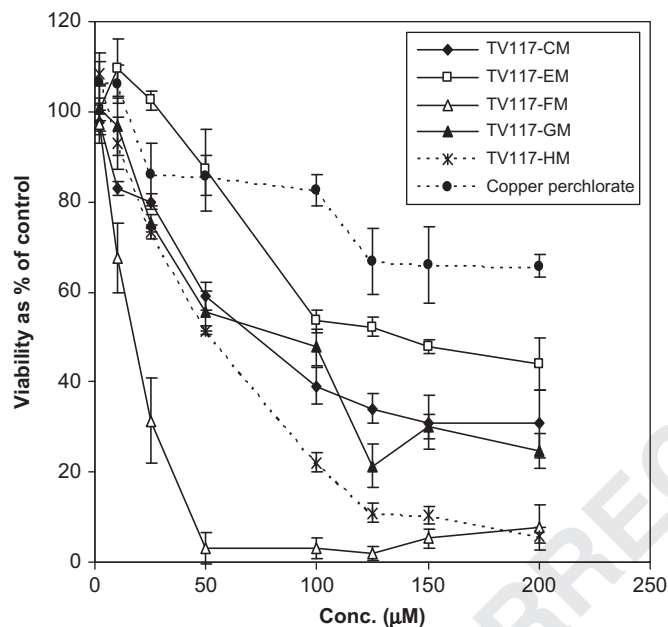

Fig. 2. Anti-proliferative effect of quinolinone Schiff base copper complexes on Hep-G_2 cells, as assessed by MTT. Results presented represent anti-proliferative assays carried out in 96 well plates with continuous exposure to assessed agent (0–200 μM) for 96 h. All assessed agents, except copper perchlorate, caused a dose-dependent decrease in cellular proliferation, with TV117-FM exhibiting the greatest potency. Results obtained were expressed as a percentage of solvent-treated control cells. Bars indicate mean ± S.E.M., $n=3$.

398.3 μM but its copper complex, TV117-FM exhibited an IC_{50} value of 17.9 μM. Moreover, TV117-FM was shown to be the compound with the greatest anti-proliferative activity in this series. Therefore, selected aspects of the mechanism of action of TV117-FM were studied.

Comparative studies were carried out using one of the most widely used Pt-based drugs, cisplatin. The cytotoxicity of cisplatin in Hep-G_2 cells was previously determined (Deegan et al., 2006). Cisplatin demonstrated an IC_{50} value of 15.0 μM in Hep-G_2 cells at 96 h (Table 1). Therefore, the anti-cancer activity seen with cisplatin was comparable to that observed with TV117-FM.

As TV117-FM was shown to be the most cytotoxic agent screened in this series, it was decided to carry out a time-course study on its cytotoxicity. For comparative purposes, the ligand, the complex and the metal salt (copper perchlorate), were included in this experiment, incubated with Hep-G_2 cells for 4, 24 and 96 h, and their effect on cellular viability determined. Results presented in Table 2 clearly indicated that the anti-proliferative activity of TV117-FM increased with time, unlike that seen with the ligand, TV117-F or copper perchlorate. Additionally, the effect of TV117-FM on cellular viability is presented in Fig. 3, where it was evident that the anti-proliferative activity was both time- and concentration-dependent.

In order to determine the possible cyto-selective nature of TV117-FM, a matched non-malignant hepatic cell line, Chang was used, as previously described in Section The model cell lines. TV117-FM exhibited a similar trend in its cytotoxicity across both cell lines after 96 h incubation, with an IC_{50} value of 17.9 μM in

Table 2
Anti-proliferative effect of TV117-F, its copper complex TV117-FM and copper perchlorate in Hep-G_2 cells following 4, 24, and 96 h continuous incubation with assessed agent in the range 0–200 μM, using MTT assay. A graph of viability (% of solvent-treated control cells) versus concentration was used to calculate the IC_{50} values (μM). Results presented are representative of thee independent experiments (Mean ± S.E.M., $n=3$). (>, greater than).

Hep-G_2 IC_{50} (μM) ± S.E.M.

Compound	4 h	24 h	96 h
TV117-F	> 200	> 200	> 200
TV117-FM	68.0 ± 12.4	26.5 ± 3.3	17.9 ± 3.8
Copper perchlorate	> 200	> 200	> 200

Table 1
Anti-proliferative effect of quinolinone Schiff bases and their corresponding copper complexes (denoted, *M*) in Hep-G_2 cells following 96 h continuous incubation with assessed agent in the range 0–1000 μM, using MTT assay. A graph of viability (% of solvent-treated control cells) versus concentration was used to calculate the IC_{50} values (μM). Results presented are representative of thee independent experiments (Mean ± S.E.M., $n=3$). (>, greater than).

Hep-G_2

Compound	Abbreviation	IC_{50} (μM) ± S.E.M.
(7E)-7-(2hydroxybenzylideneamino)-4-methylquinolin-2(1H)-one	**TV117-C**	411.6 ± 7.3
(7E)-7-(2,3dihydroxybenzylideneamino)-4-methylquinolin-2(1H)-one	**TV117-E**	> 1000
(7E)-7-(3-ethoxy-2-hydroxybenzylideneamino)-4-methylquinolin-2(1H)-one	**TV117-F**	398.3 ± 33.2
(7E)-7-(2-hydroxy-3-methoxybenzylideneamino)-4-methylquinolin-2(1H)-one	**TV117-G**	175.7 ± 12.2
(7E)-7-(2-hydroxy-4-methoxybenzylideneamino)-4-methylquinolin-2(1H)-one	**TV117-H**	521.7 ± 4.7
[Cu(^{a}L)$_2$]-CM	**TV117-CM**	74.8 ± 8.9[b]
[Cu(^{a}L)$_2$]-EM	**TV117-EM**	129.0 ± 16.3[b]
[Cu(^{a}L)$_2$]-FM	**TV117-FM**	17.9 ± 3.8[b]
[Cu(^{a}L)$_2$]-GM	**TV117-GM**	105.3 ± 25.4[b]
[Cu(^{a}L)$_2$]-HM	**TV117-HM**	53.2 ± 1.6[b]
Copper perchlorate	**CuClO$_4$**	> 200
cis-diamminedichloroplatinum(II)	**Cisplatin**	15.0 ± 2.6

[a] **L** denotes ligand.
[b] $P < 0.01$ statistically different from IC_{50} value of parent ligand).
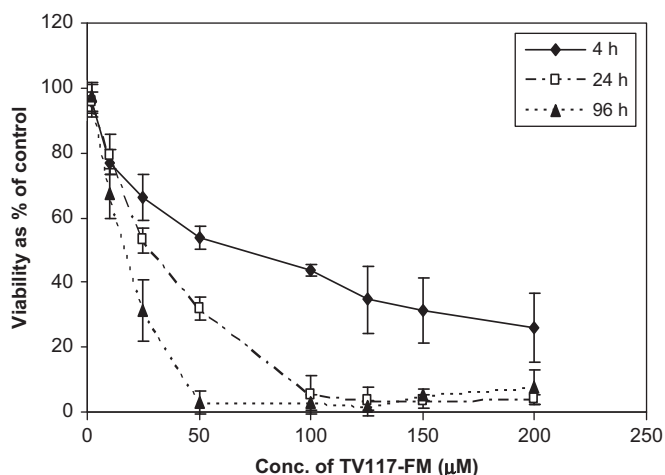
Fig. 3. Anti-proliferative effect of TV117-FM on Hep-G_2 cells over time, as assessed by MTT. Results presented represent anti-proliferative assays carried out in 96 well plates with continuous exposure to assessed agent (0–200 μM) for 4, 24 or 96 h. TV117-FM caused both a concentration- and time-dependent dependent decrease in cellular proliferation. Results obtained were expressed as a percentage of solvent-treated control cells. Bars indicate mean ± S.E.M., $n=3$.
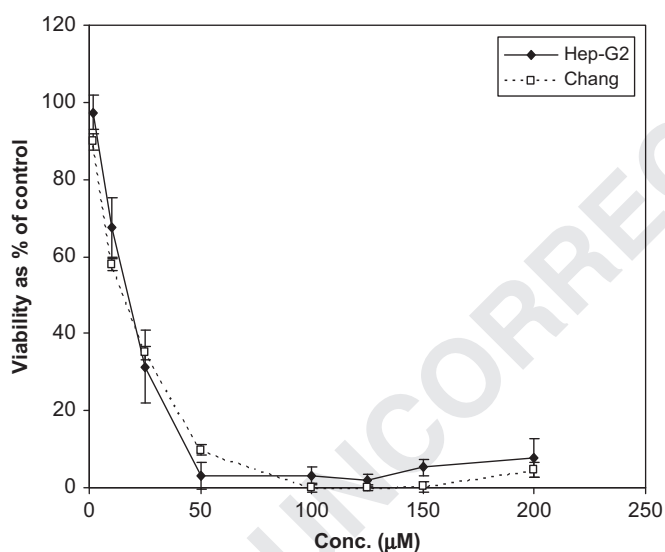

Fig. 4. Anti-proliferative effect of TV117-FM on Hep-G_2 and Chang cells following 96 h treatment, as assessed by MTT. Results presented represent anti-proliferative assays carried out in 96 well plates with continuous exposure to assessed agent (0–200 μM) for 96 h. TV117-FM exhibited similar cytotoxicity trends between the hepatic cell carcinoma-derived Hep-G_2 cells and the Chang cells (derived from normal liver tissue). IC_{50} values were found to be 17.9 and 15.3 μM in Hep-G_2 and Chang cells, respectively. Results obtained were expressed as a percentage of solvent-treated control cells. Bars indicate mean ± S.E.M., $n=3$.

Hep-G_2 cells and an IC_{50} value of 15.3 μM in Chang cells (Fig. 4). These data suggested that TV117-FM was cytotoxic to both cell lines and did not offer cyto-selectivity, in the model used.

To determine whether TV117-FM was a potential substrate for P-gp-mediated MDR, both CHC5 and CHO-K1 cells were incubated for 96 h with a range of concentrations of assessed agent. Subsequently, the IC_{50} was determined by MTT assay and used to calculate resistance factors (Rf) for both cell lines (Table 3). Vinblastine, a known P-gp substrate exhibited lower anti-proliferative activity in CHC5 cells than in CHO-K1 cells, due to its efflux from CHC5 cells via P-gp. Rf's were calculated to determine the fold-difference in concentration of assessed agent, required to elicit the same effect (IC_{50}) in the CHC5 cells than in the CHO-K1

Table 3
Anti-proliferative effect of TV117-FM in P-gp deficient CHO-K1 cells and P-gp over-expressing CHC5 cells following 96 h continuous incubation with assessed agent, using MTT assay. The resistance factor (Rf) was calculated using vinblastine, a known P-gp substrate. Rf for assessed agents were also calculated and compared to that of vinblastine to demonstrate whether assessed agents were substrates for P-gp. Results presented are representative of thee independent experiments (Mean ± S.E.M., $n=3$).

	IC_{50} (μM) ± S.E.M.		
Compound	**CHO-K1**	**CH^{R}C5**	**Rf**
Vinblastine	0.01 ± 0.005	0.475 ± 0.10	47.5
TV117-FM	16.90 ± 1.02	54.67 ± 10.17	3.2

cells. The Rf for vinblastine was 47.5. Therefore, an assessed agent with an Rf equal to or greater than 47.5 suggested that they were likely to be effluxed from cells in a manner similar to that seen with the known P-gp substrate, vinblastine. However, TV117-FM possessed an Rf of 3.2, which was a factor of 15 lower than that seen with vinblastine. Thus, TV117-FM was less likely to be a substrate for P-gp-mediated MDR, than vinblastine.

3.2. Mutagenic potential of assessed agents

The Standard Ames test was employed in order to establish the mutagenic potential of TV117-F, TV117-FM and copper perchlorate, along with their phase I metabolites. Positive controls consisting of nitro-quinoline-N-oxide (NQNO) for TA98 and sodium azide (NaN$_3$) for TA100 were included in all experiments (data not shown). Furthermore, the activity of the S9 fraction was validated with the inclusion of 2-aminoanthacene, which requires metabolic activation prior to producing a positive mutagenic response in the Ames test (data not shown).

The results presented in Figs. 5 and 6 indicated that none of the assessed agents along with their phase I metabolites, caused mutation by either frame-shift or base-pair substitution, as no concentration-dependent fold-increase in revertant colonies was apparent. Furthermore, each assessed agent was screened at concentrations over four orders of magnitude, in an effort to visualise any possible mutagenic effect. Therefore, using the Standard Ames test neither the ligand, its copper complex nor copper perchlorate met the criteria, and so cannot be considered mutagenic.

3.3. Determination of the intercalative nature of TV117-FM

The ability of assessed agents to intercalate DNA, thus contributing to decreased cell viability, was elucidated using gel mobility shift assays. Plasmid DNA was incubated with assessed agent over a range of concentrations equivalent to the IC_{25}, IC_{50} and IC_{75} (as determined using Hep-G_2 cells following 96 h) and DNA was subsequently separated using agarose gel electrophoresis. Doxorubicin, a known DNA intercalator was used as a positive control (25 μM). The results obtained (Fig. 7) clearly indicated that regardless of concentration used (9–30 μM) TV117-FM did not intercalate DNA, as it did not alter the migration pattern of either open circular (OC) or supercoiled (SC) conformations of plasmid DNA, unlike the positive control, doxorubicin.

3.4. Inhibition of DNA synthesis

In an attempt to determine the effect of assessed agents on DNA synthesis, BrdU incorporation assays were carried out, according to the method described by Portsmann et al. (1985). These assays were carried out in Hep-G_2 cells following 4, 24 and
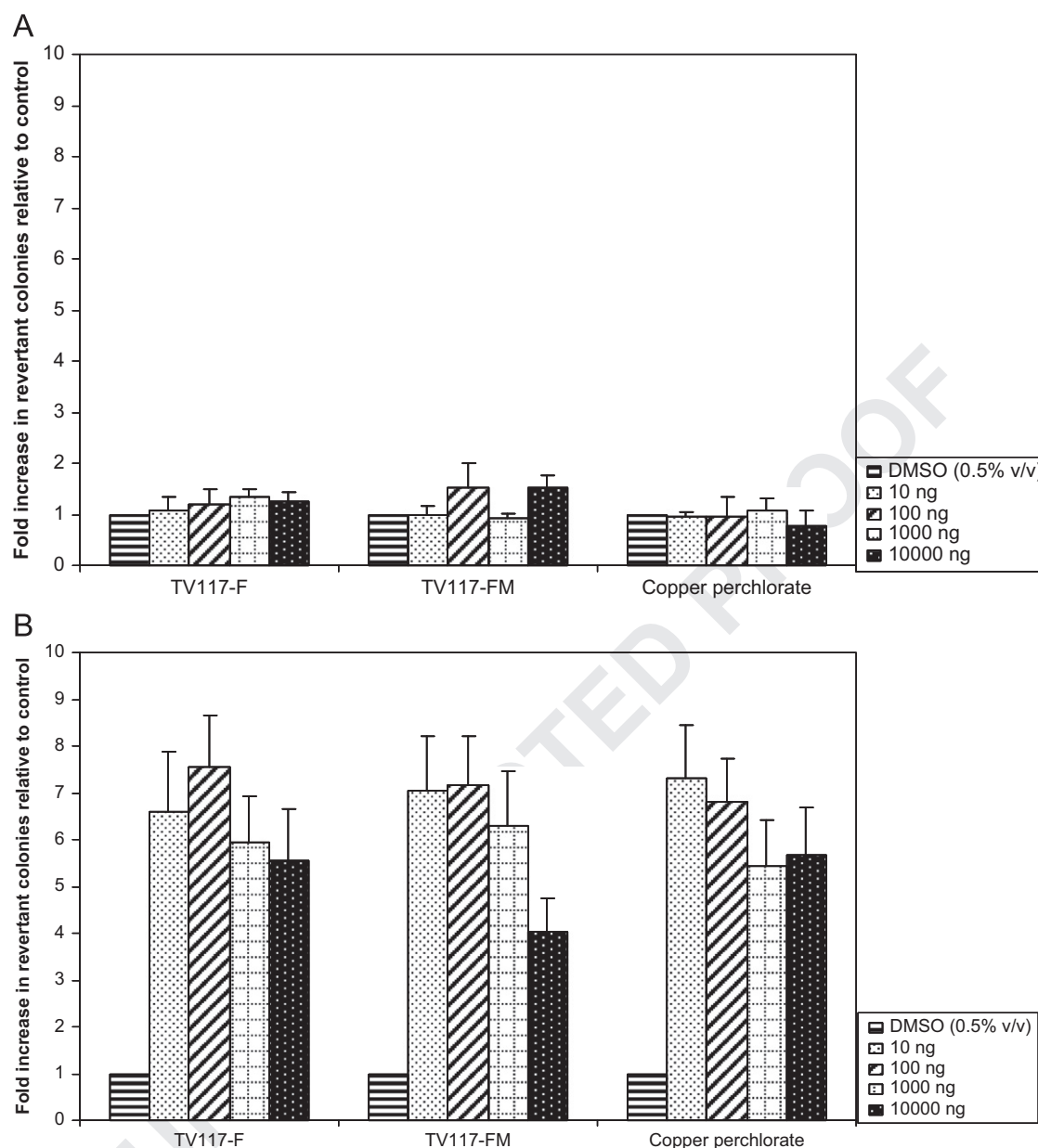
Fig. 5. Effect of TV117-F, its copper complex TV117-FM and copper perchlorate on *S. typhimurium* strain TA98 in the (A) absence and (B) presence of a mammalian metabolic activation system (S9). Results presented represent Standard Ames assay carried out following 72 h continuous exposure of TA98 to assessed agent. Results obtained were expressed as a fold increase in revertant colonies relative to control. Bars indicate mean ± S.E.M., $n = 3$.

96 h incubation with assessed agent. Additionally, actinomycin D was included in experiments as a positive control (Fig. 8B). TV117-FM exhibited both a time- and concentration-dependent decrease in DNA synthesis by 96 h and at high concentrations, caused a complete inhibition of DNA synthesis (Fig. 8A). Moreover, this response was similar to that of actinomycin D (Fig. 8B).

3.5. Inhibition of topoisomerase II

Quinolinones have been shown to inhibit prokaryotic DNA cleavage via inhibition of topoisomerase IV and DNA gyrase (Wang et al., 2008). The eukaryotic homolog of topoisomerase IV is topoisomerase II. Therefore, the ability of each assessed agent to inhibit topoisomerase II mediated relaxation of pGEM-3Z plasmid DNA was determined (see Section DNA topoisomerase II strand-passing assay). Electrophoretic analysis (Fig. 9) demonstrated that there was a low proportion of OC together with a higher proportion

of SC DNA in the control sample, which did not contain enzyme or inhibitor (Fig. 9, lane 2). Incubation of pGEM-3Z DNA with topoisomerase II resulted in the formation of a significant proportion of relaxed DNA bands and a reduction in the bands representative of OC and SC DNA (Fig. 9, lane 3). The positive control novobiocin (10 μM) inhibited the activity of topoisomerase II (Fig. 9, lane 4), while TV117-F (200 μM) was capable of inhibiting topoisomerase II activity (Fig. 9, lane 9) in a manner similar to that seen with the positive control (Fig. 9, lane 4). However, none of the other assessed agents, and at any of the concentrations used, displayed comparable inhibition of TopoII activity (Fig. 9, lanes 5–8, lane 10).

4. Discussion

The aim of the present study was to determine the in vitro cytotoxic potential of novel quinolinone Schiff base ligands and
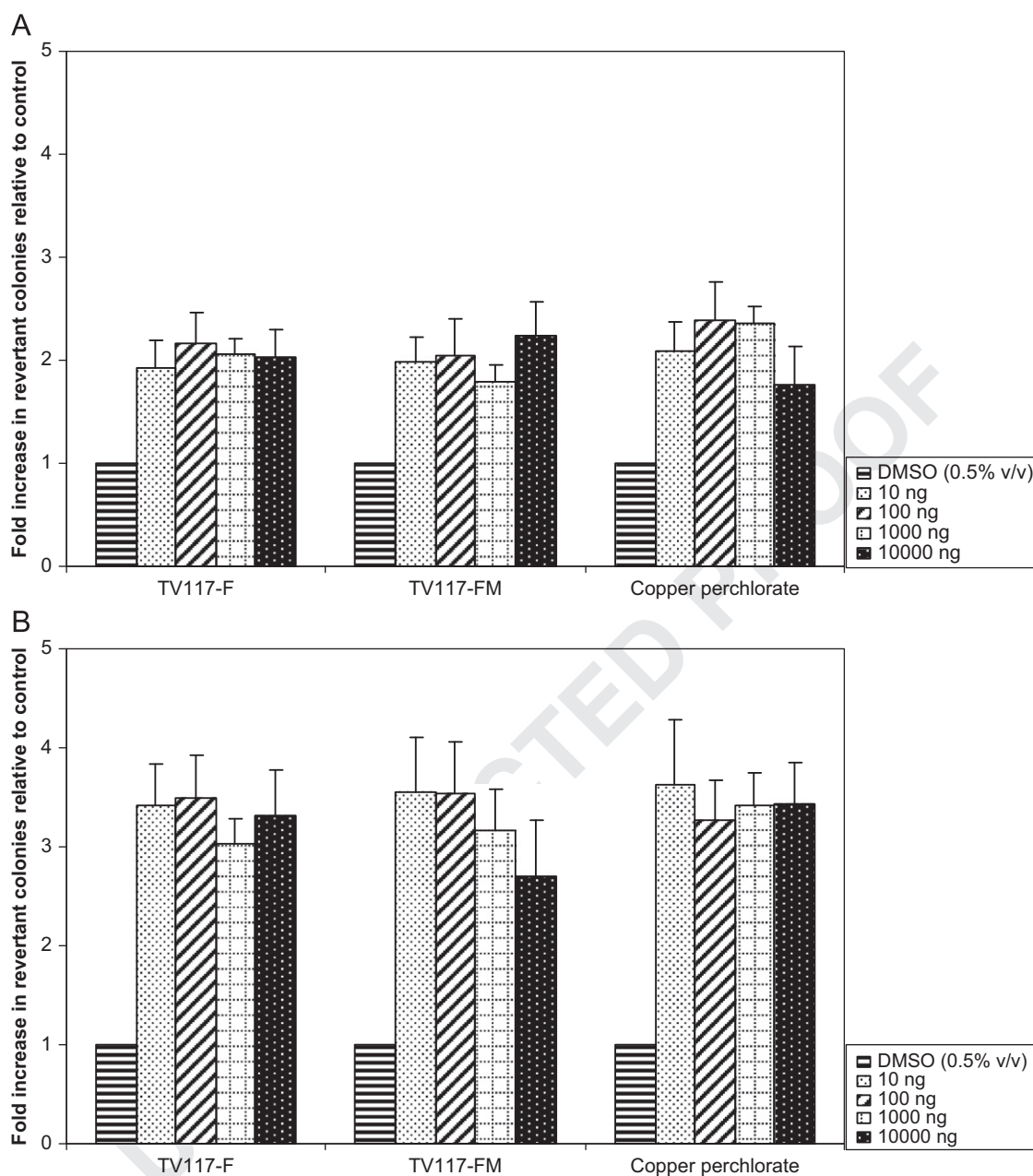
Fig. 6. Effect of TV117-F, its copper complex TV117-FM and copper perchlorate on *S. typhimurium* strain TA100 in the (A) absence and (B) presence of a mammalian metabolic activation system (S9). Results presented represent Standard Ames assay carried out following 48 h continuous exposure of TA100 to assessed agent. Results obtained were expressed a as fold increase in revertant colonies compared to control. Bars indicate mean ± S.E.M., $n = 3$.

their copper(II) complexes, along with elucidating the cyto-selective, mutagenic and TopoII inhibitory potential of the most potent assessed agent(s). Presently, treatment for HCC may involve surgical resection, transplantation, TACE or radioemboli-sation with the treatment modality dependent upon staging of the disease (Wörns and Galle, 2010). More recently, the use of targeted therapies, such as the tyrosine kinase inhibitor sorafenib, and the somatostatin analogue octreotide, have proved to be promising in the treatment of advanced stage HCC, both singly and in combination (Del Prete et al., 2010; Caraglia et al., 2011; Marra et al., 2011). In addition, Caraglia and co-workers (2011) reported a gradual decrease in ROS production and ERK1/2 activation in patients responsive to treatment with sorafenib plus octreotide, over a 21-day period. These authors suggested that ROS and activated ERK1/2 status in peripheral blood mononuclear cells may be used as predictors of response to treatment with such targeted agents.

It has previously been shown that transition metal complexes of Schiff base ligands can improve the anti-microbial and anti-cancer activities of the ligand (Holla et al., 2008). More specifi-cally, several Schiff bases ligands and their copper(II) complexes have enhanced anti-neoplastic activity (Zhao et al., 2007; Barraja et al., 2010, 2011). Our research group has previously described the potent anti-cancer activity and the in vitro mechanisms of action of a series of innovative copper-, silver- and manganese-complexes of phen and phendione ligands (Deegan et al., 2006, 2007).

Here anti-proliferative assays using MTT were carried out to determine IC_{50} values for each quinolinone Schiff base and their corresponding copper(II) complexes in Hep-G_2 cells following
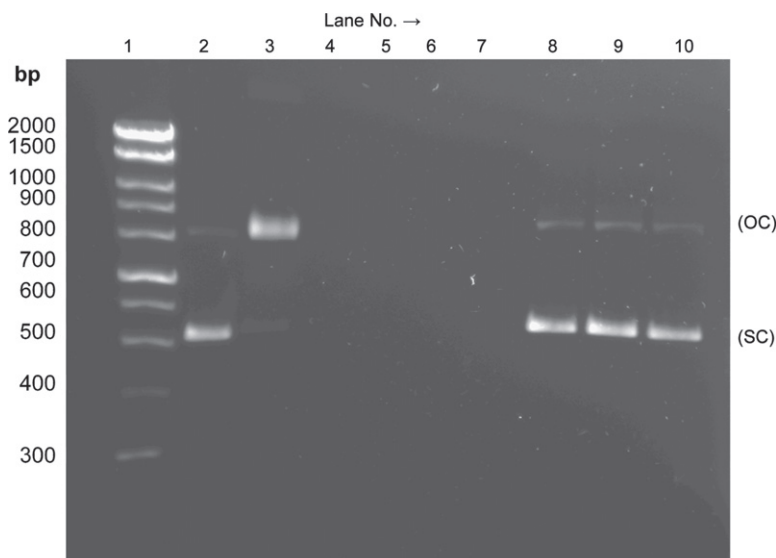
Lane No:	Treatment	Lane No:	Treatment
1	MW Markers	6	Vacant
2	DMSO (0.25 %, v/v)	7	Vacant
3	doxorubicin (25 μM)	8	TV117-FM (9 μM)
4	Vacant	9	TV117-FM (17.9 μM)
5	Vacant	10	TV117-FM (30 μM)

Fig. 7. Effect of TV117-FM on the electrophoretic migration of pGEM-3Z plasmid DNA. Purified pGEM-3Z plasmid DNA (0.05 μg/μl) was incubated with increasing concentrations of TV117-FM for 2 h. Reactions were analysed by agarose gel electrophoresis and stained with ethidium bromide. TV117-FM did not appear to inhibit the migration of open circular (OC) or supercoiled (SC) conformations of plasmid DNA, regardless of concentration, indicating it does not intercalate DNA. Experiments were carried out in triplicate, results presented represent those obtained from a single experiment.

96 h incubation. The copper salt; copper perchlorate (CuClO$_4$) was also included in assays in order to determine if inhibition in cellular viability was caused by the ligand, the complex or the simple salt alone. Moreover, results obtained were compared to the anti-proliferative effect of cisplatin seen previously, in this cell line (Deegan et al., 2006). These assays showed that following 96 h incubation, quinolinone Schiff bases exhibited relatively poor anti-proliferative effects (Table 1; Fig. 1). However, cytotoxicity was enhanced in all corresponding copper complexes (Table 1; Fig. 2). Additionally, CuClO$_4$ proved to be inactive as an independent anti-cancer agent. Therefore, the anti-proliferative effect seen was significantly enhanced by complexation of the quinolinone Schiff bases to copper. The compound exhibiting the greatest cytotoxicity was TV117-FM. Moreover, this complex displayed an anti-neoplastic profile similar to that seen with cisplatin.

Subsequently, the time-dependent anti-cancer activity of TV117-FM, its parent ligand; TV117-F and the copper salt; CuClO$_4$ was determined. TV117-FM displayed both a concentration- and time-dependent decrease in cellular proliferation (Table 2; Fig. 3) which was in contrast to that seen for TV117-F and CuClO$_4$. Moreover, TV117-FM exhibited a greater anti-proliferative effect 4 h post-treatment compared to the Schiff bases studied. In addition, following 96 h incubation of Hep-G$_2$ cells with TV117-FM, a near-complete inhibition of metabolic activity was observed at concentrations greater than 50 μM (Fig. 3). Therefore, TV117-FM appeared to be a potent, fast-acting, novel anti-cancer agent, compared to its parent ligand or indeed any ligand screened in the series.

An extremely rare attribute of anti-cancer agents is their ability to naturally select and inhibit the growth of neoplastic over non-neoplastic cells. Thus, to ascertain whether TV117-FM possessed such a trait, its cyto-selective nature was determined using Chang cells. These cells were thought to be derived from normal hepatic tissue, therefore, they are a commonly used match to assess the cyto-selectivity of agents already screened with Hep-G$_2$ cells (Deegan et al., 2006, 2007). However, it should be noted that Chang cells have known HeLa cell contamination, therefore any potential data obtained indicating cyto-selectivity towards these model cells should be confirmed by screening using primary human hepatocytes. Results presented in Fig. 4 suggested that TV117-FM was incapable of selecting malignant over non-malignant targets, in a trend dissimilar to that seen previously with cisplatin (Deegan et al., 2006). However, as many potent therapeutics are incapable of displaying cyto-selectivity, it was decided to further evaluate the anti-cancer activity of this novel agent.

In order to ensure that the anti-proliferative effect seen with TV117-FM was not attenuated by P-gp mediated MDR, a cellular model for MDR-1 was used. Comparison of the anti-proliferative data from the CHO-K1 and CHC5 cells indicated that TV117-FM was not a likely substrate for P-gp compared to the known P-gp substrate, vinblastine (Table 3). Furthermore, TV117-FM was shown to be 15 times less likely to be effluxed from a P-gp abundant cell relative to vinblastine. Thus, it is unlikely that TV117-FM is a substrate for P-gp mediated MDR and therefore remains within cells where its cytotoxic potential may ensue.

Additionally, it is desirable that novel anti-cancer agents are non-mutagenic, as it would be unfavourable to administer a chemotherapeutic compound which in itself may cause mutations. For this reason, our assessed agents were analysed using the Standard Ames test to determine their mutagenic potential. The results obtained (Figs. 5 and 6) showed that TV117-F, TV117-
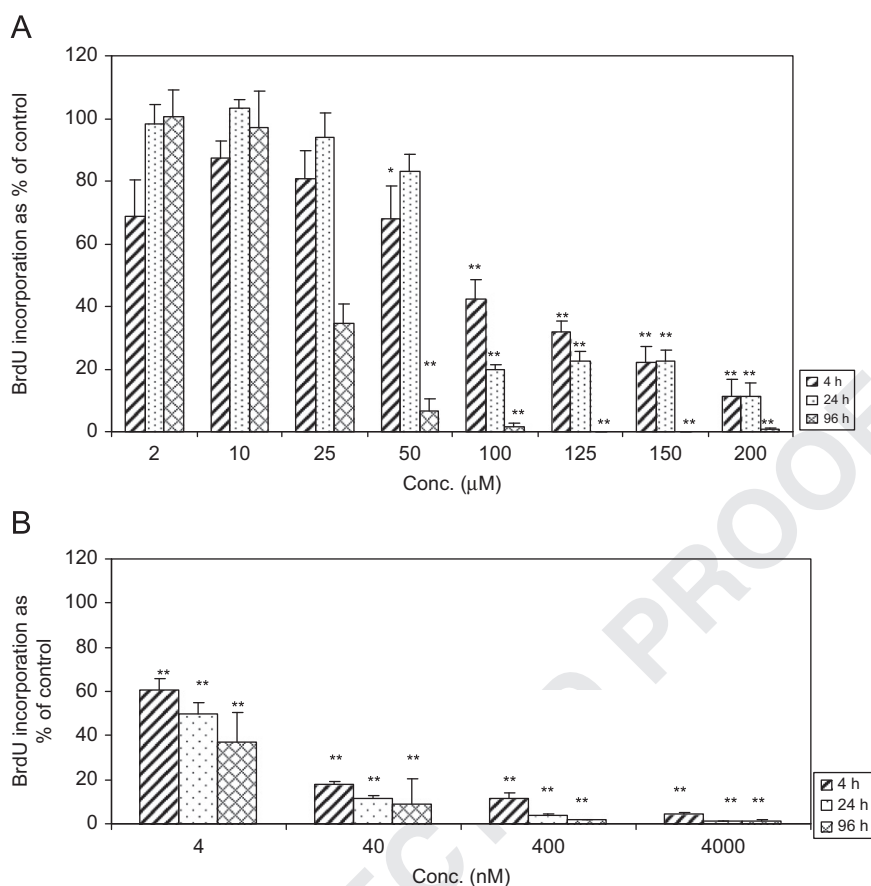
Fig. 8. Effect of (A) TV117-FM and (B) Actinomycin D on DNA synthesis in Hep-G$_2$ cells, as assessed by BrdU incorporation. Results presented represent BrdU incorporation assays carried out in 96 well plates with continuous exposure to assessed agents (0–200 μM) for 4, 24 and 96 h. (A) TV117-FM and (B) Actinomycin D caused a time- and dose-dependent decrease in DNA synthesis. Results obtained were expressed as BrdU incorporation as a percentage of solvent-treated control cells. Bars indicate mean ± S.E.M., $n=3$. *$P < 0.05$ **$P < 0.01$.

FM and CuClO$_4$ did not cause a significant dose-dependent increase in the number of revertant colonies, regardless of tester strain used, and the presence or absence of the S9 enzyme activation system. This suggested that these agents and their phase I metabolites were non-mutagenic. These results compare favourably with other copper complexes previously examined by our group using the Standard Ames test (Deegan et al., 2006, 2007). Here, copper-phendione and copper-phen complexes, along with their phase I metabolites were shown to be non-mutagenic. Thus, the current findings indicated that if TV117-FM was used clinically, its application would not be limited by its potential mutagenicity, unlike that seen with cisplatin (Hannan et al., 1989; Cross et al., 1996). From their studies they highlighted the possible risk of secondary malignancies in patients undergoing treatment with cisplatin.

In an effort to determine if TV117-FM inhibited DNA synthesis directly by targeting the DNA molecule, in a manner analogous to cisplatin, electrophoretic mobility shift assays were employed. Results presented in Fig. 7 confirmed that, regardless of the concentration used, TV117-FM did not intercalate DNA, unlike the positive control, doxorubicin. Therefore, TV117-FM ultimately inhibited DNA synthesis although it was by a mechanism other than intercalation and the exact mechanism remains to be determined.

Having established that TV117-FM did not intercalate DNA, we next sought to examine its effect on DNA synthesis, using BrdU incorporation assays. The results obtained suggested that TV117-FM exhibited significant inhibitory effect which was both time- and concentration-dependent, in a trend similar to that seen with

the positive control, actinomycin D. Furthermore, these results are consistent with those seen by Deegan et al. (2006, 2007). These researchers reported a concentration-dependent inhibition of DNA synthesis in Hep-G$_2$ cells by copper-phendione and copper-phen agents.

The prevention of de novo replication of DNA via topoisomerase II inhibition is a desirable characteristic of any novel chemotherapeutic agent (Larsen et al., 2003). Additionally, many quinolinone-, quinoline- and coumarin-based compounds have been shown to be potent topoisomerase II inhibitors (Bredberg et al., 1991; Corbett et al., 1993; Lorico and Long, 1993; Finn et al., 2004). Therefore, it was decided to examine the effect of TV117-FM, its quinolinone ligand, TV117-F and CuClO$_4$ on the inhibition of topoisomerase II activity. These experiments were conducted using a cell-free system (see Section DNA topoisomerase II strand-passing assay). Results presented in Fig. 9 indicated that TV117-F was the only assessed agent capable of inhibiting topoisomerase II activity and in a manner similar to the positive control, novobiocin. This result indicated that when TV117-F was complexed with copper, its ability to interact with topoisomerase II was diminished. Additionally, TV117-FM caused a dose-dependent decrease in DNA synthesis, although, this effect was not mediated though DNA intercalation (Fig. 7). Therefore, from the data presented here, it was clear that the presence of a copper moiety in the quinolinone Schiff base structure somehow prevented inhibition of topoisomerase II. However, the exact mechanism(s) underlying this process remains to be elucidated.

In conclusion, the in vitro cytotoxic, cyto-selective and mutagenic potential of a series of novel quinolinone Schiff base ligands
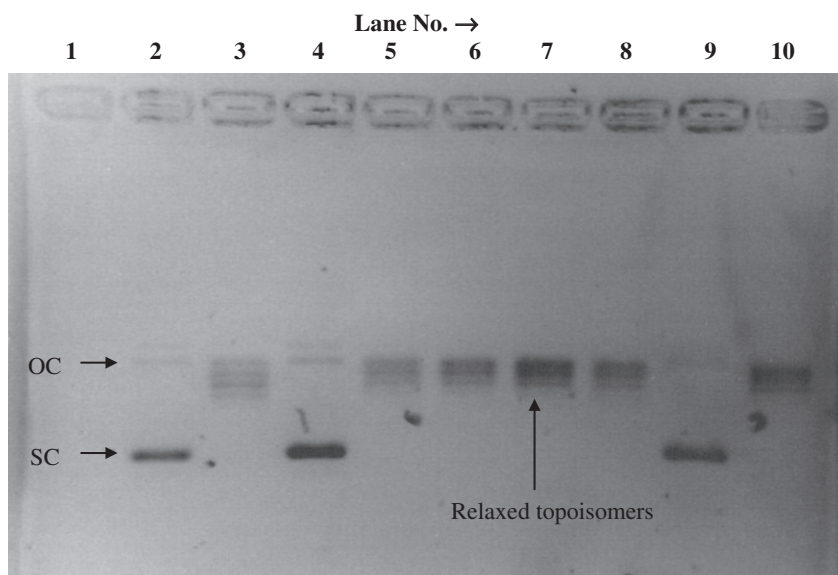
Lane No. →

Lane No:	Treatment	Lane No:	Treatment
1	Vacant	6	TV117-FM (17.9 μM)
2	pGEM-3Z DNA	7	TV117-FM (30 μM)
3	pGEM-3Z DNA + Topo II	8	CuClO₄ (200 μM)
4	novobiocin (10 μM)	9	TV117-F (200 μM)
5	TV117-FM (9 μM)	10	DMSO [0.25 % (v/v)]

Fig. 9. Effect on topoisomerase II mediated relaxation of pGEM-3Z plasmid DNA. Purified pGEM-3Z plasmid DNA (0.05 μg/μl) was incubated with topoisomerase II alone or along with assessed agent for 30 min. Reactions were analysed by agarose gel electrophoresis and stained with ethidium bromide. TV117-F (lane 9) appeared to inhibit topisomerase II activity in a manner similar to that of the positive control novobiocin (lane 4). Experiments were carried out in triplicate, results presented represent those obtained from a single experiment.

and corresponding Cu(II) complexes have been studied. The most potent Cu complex was TV117-FM, an agent unlikely to be effluxed from cells via P-gp, dissimilar to many chemotherapeutic agents. Furthermore, it inhibited DNA synthesis in a concentration- and time-dependent manner, but not though intercalation or inhibition of topoisomerase II. Taken together, the work presented here culminated in identification of a potent anti-cancer agent, with a cytotoxic profile similar to the clinically used anti-cancer drug, cisplatin. Future studies will focus on identifying the possible pro-apoptotic mechanisms underlying the anti-proliferative effects of TV117-FM. Additionally, it is intended that proteomic studies may allow identification of key cellular targets and in doing so, assist in elucidating their mechanisms of action, thus facilitating the development of an effective anti-cancer agent for the treatment of HCC.

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