Monofunctional Platinum-containing Pyridine-based Ligand Acts Synergistically in Combination with the Phytochemicals Curcumin and Quercetin in Human Ovarian Tumour Models

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Abstract. *With the idea that platinum compounds that bind with DNA differently than cisplatin may be better-able to overcome platinum resistance in ovarian tumor, the monofunctional platinum complex tris(imidazo(1,2-α)pyridine) chloroplatinum(II) chloride (coded as LH6) has been synthesized and investigated for its activity, alone and in combination with the phytochemicals curcumin and quercetin, against human ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cancer cell lines. LH6 is found to be more active than cisplatin against the resistant cell lines and its bolus combinations with curcumin and quercetin are found to produce more pronounced cell kill. Whereas platinum accumulation from cisplatin is found to increase almost linearly with time, that from LH6 reaches a maximum at 4 h and is somewhat lowered at 24 h. It is possible that the presence of bulky hydrophobic imidazo (1,2-α-pyridine) ligand in LH6 facilitates its rapid uptake through the cytoplasmic membrane. Lower platinum accumulation at 24 h than at 4 h for LH6 can be seen to imply that efflux processes may be more dominant as the period of incubation is increased. When platinum-DNA binding levels at 24 h are compared, cisplatin is found to be associated with the higher level in the parent A2780 cell line and LH6 in the resistant A2780^{cisR} cell line, in line with greater activity of cisplatin in the parent cell line and that of LH6 in the resistant cell line. If the observed in vitro activity of LH6 is confirmed in vivo, it can be seen to have the potential for development as novel platinum based anticancer drug.*

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Ovarian cancer is dubbed as the silent killer in women, mainly because of its detection at an advanced stage when it is likely to have metastasized beyond the ovaries. Most patients die of recurrence of the disease that develops resistance to conventional treatment (1, 2). Although combination of cisplatin (and carboplatin) with paclitaxel forms the standard choice of chemotherapy, platinum-resistance remains a major obstacle in the treatment of epithelial ovarian cancer (3). Hence, there is need for new platinum-based drugs having novel mechanisms of action and the use of different treatment regimens.

Recently, it has been shown that monofunctional cationic platinum complexes with only one site for binding to DNA can have a greater efficacy than both cisplatin and oxaliplatin in killing cancer cells and inhibiting transcription (4-6). In this study, we report the synthesis of a new monofunctional platinum(II) complex having the general formula [PtL$_3$Cl]Cl, where L=imidazo(1,2-α)pyridine [coded as LH6], and its activity alone and in combination with phytochemicals curcumin and quercetin against human ovarian A2780, A2780^{cisR} and A2780^{ZD0473} cancer cell lines. The interaction of LH6 with salmon sperm DNA and pBR322 plasmid DNAs have also been investigated. The cytotoxicity of cisplatin has also been determined to serve as a reference. Since the molecular mechanisms of action of LH6 and the phytochemicals are likely to be different, it is thought that they may act synergistically in combination (7-9).

Curcumin is a polyphenol from rhizome of the plant *Curcuma longa* (Figure 1) that has antioxidant, anti-inflammatory, cancer chemopreventive and potentially chemotherapeutic properties (1, 8). Recent studies have demonstrated that diverse activities of curcumin in terms of antioxidant, antiproliferative and antiangiogenic functions are mediated through multiple signalling pathways (10) and that the molecular targets of curcumin include transcription factors, growth factors, cytokines, enzymes and other gene products (11). Curcumin has been shown to inhibit the Fanconi anaemia/breast cancer (FA/BRCA) pathway and sensitize ovarian cancer cells (CAOV3 and SKOV3) towards cisplatin-induced apoptosis (12, 13).

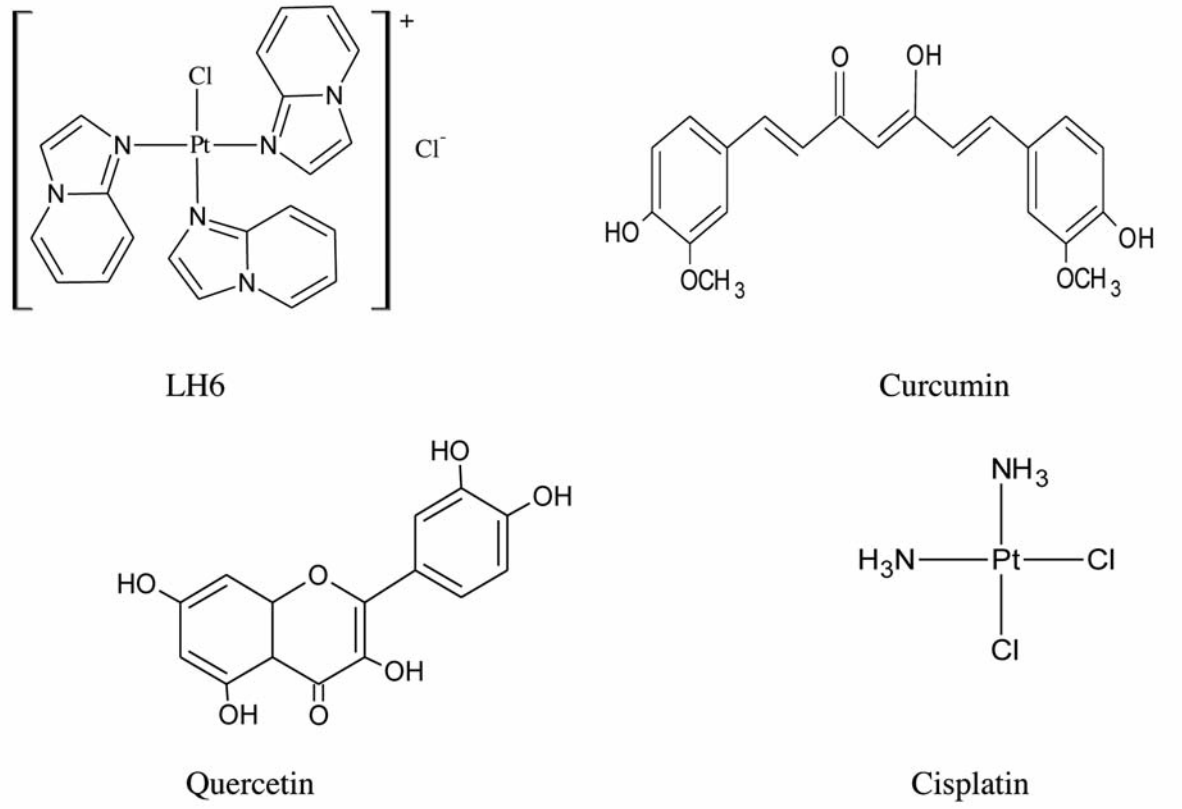

Figure 1. *Structures of LH6, curcumin, quercetin and cisplatin.*

Quercetin is a polyphenol that is chemically classified as a flavonoid (Figure 1). Ubiquitously present in fruits, such as cranberries, elderberries and red apples, vegetables, such as onions, seeds, nuts and tea (14, 15), quercetin is a well-known antioxidant that displays a variety of biological activities, including chemoprevention and inhibition of tumor growth (16). Quercetin strongly increases intracellular reactive oxygen species (ROS) levels by producing quercetin radicals (Quercetin-O•) after peroxidase-catalyzed oxidation during scavenging reactive peroxyl radicals (17), thus causing free radical-induced apoptosis through ROS/ AMPKα1/ ASK1/p38 and the AMPKα1/COX2 signalling pathways (18). Quercetin radicals are also found to lower intracellular glutathione (GSH) pool in a concentration-dependent manner (18) and trigger apoptosis through mitochondrial depolarization (19). Quercetin is known to interact with type II estrogen binding site. It is reported that quercetin acts synergistically with cisplatin in the killing of ovarian cancer cells, such as OVCA433, CAOV3 and SKOV3 (1).

Materials and Methods

Synthesis and characterization. Materials. Potassium tetrachloroplatinate (II) ($K_2[PtCl_4]$) (Sigma Chemical Company St. Louise, abbreviated state, USA); N,N-dimethylformamide [DMF] [C_3H_7NO]; imidazo(1,2-α)pyridine [$C_7H_6N_2$] (Sigma Aldrich Chemical Company, Milwaukee, MO, USA); HCl (Ajax chemicals, Auburn NSW, Australia); ethanol (Merck Pty.Ltd., Kilsyth, Australia).

Method. 1 mmol of K_2PtCl_4 (0.415 g) was dissolved in milli-Q (mQ) water (10 ml) and treated with concentrated hydrochloric acid (0.25 ml). The temperature of the solution was increased to 50˚C. Eight millimoles of imidazo(1,2-α)pyridine (0.811 ml, 0.945 g) were added dropwise over of 1 h to the solution of potassium tetrachloroplatinate. The reaction mixture was stirred at room temperature for 2 weeks. Twenty ml of 0.25 M hydrochloric acid was added to the mixture and stirring was continued for another week at room temperature. The mixture was centrifuged at 5,500 rpm for 10 min to collect precipitate of LH6. The crude product of LH6 was washed with ice cold ethanol and purified by precipitation from 0.05 M HCl. The yield of the product was 46% (291 mg) as a pale yellow powder.

Characterization. C, H and N were determined using a Carlo Erba 1106 automatic analyzer available at the Australian National University. Pt was determined by graphite furnace atomic absorption spectroscopy (AAS). As LH6 could not be obtained in crystalline form, infrared spectrometry (IR), mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H NMR) spectra were used to aid in its structural characterization. The IR spectrum was obtained using a Varian FT-IR spectrometer (Bruker IFS66 spectrometer;

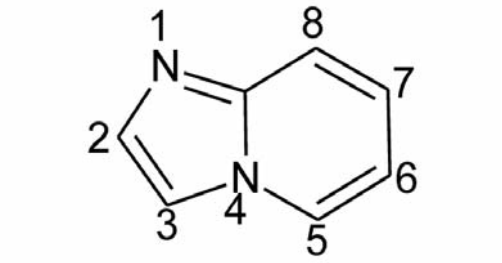

Figure 2. *Numbering scheme adopted for imidazo(1,2-α)pyridine ligand present in LH6.*

Maker address). To obtain the mass spectrum, a solution of LH6 made in 90% methanol and 10% dimethylformamide (DMF) was sprayed into a Finnigan LCQ mass spectrometer (Maker address). To obtain 1H NMR spectrum, the compound was dissolved in deuterated DMSO (dimethyl sulfoxide) and prepared in 5 mm high precision Wilmad NMR tube and a Bruker DPX400 spectrometer was used with frequency of 400.2 MHz. In 1H NMR, s, d and q denote respectively singlet, doublet and quartet. The numbering scheme adopted for imidazo(1,2-α)pyridine ligand which is given below in Figure 2:

LH6. As a pale yellow powder (291 mg, 46 %); ^{1}H NMR (400 MHz, [D_6] DMSO: δ (ppm)=9.3 (d, due to C_2H), 8.8 (d, due to C_3H), 8.5 (d, due to C_5H), 8.1 (d, due to C_4H), 7.8 (q, due to C_7H), 7.4 (q, due to C_8H); 3.8 (s, due to water), 2.5 (s, due to DMSO); IR (KBr): 3109, 1634, 1505, 1314, 1235, 1167, 1143, 757 and 623 cm^{-1}; MS (ESI) *m/z* (%):584.93 (100)=[Pt($C_7H_6N_2$)$_3$$Cl_2$-Cl]; 583.93 (100)=[Pt($C_7H_6N_2$)$_3$$Cl_2$-Cl-H]; 333.53 (90)=[Pt($C_7H_6N_2$)$_3$$Cl_2$-($C_7H_6N_2$)$_2$-Cl-(-$NH_2$)-H]; 583.93 (100)=[Pt($C_7H_6N_2$)$_3$$Cl_2$-Cl-2H]; 334 (55)=[Pt($C_7H_6N_2$)$_3$$Cl_2$-($C_7H_6N_2$)$_2$-Cl-(-$NH_2$)]; Anal. calcd for $C_{21}H_{18}Cl_2N_6Pt$: C 40.6, H 3.4, N 13.5, Pt 31.3, found: C 40.1±0.4, H 3.6±0.4, N 13.4±0.4, Pt 31.5±1.0.

Molar conductivity. Molar conductivity values for solutions of LH6 and cisplatin (CS) were determined at concentrations ranging from 60 μM to 200 μM. First, to obtain 1 mM solutions, CS was dissolved in 1:4 mixture of DMF and mQ water, and LH6 in 2:1 mixture of DMSO and mQ water; these were then further diluted with mQ water. The molar conductivity values were plotted against concentration to obtain limiting molar conductivity values (Λ_o).

Biological activity. Interaction with DNA. Interaction of cisplatin and LH6 with salmon sperm DNA and pBR322 plasmid DNA (without and with BamH1 digestion) were investigated using agarose gel electrophoresis in which the amount of DNA was kept constant, while concentrations of the compounds were varied. DNA bands were viewed under short wave UV light and a Kodak Gel Logic 100 imaging system (GL 100) was used for taking gel pictures. Images were analyzed using Kodak molecular imaging software (Kodak MI software).

Salmon sperm DNA (ssDNA). To prepare stock solutions of ssDNA, 10-15 mg of ssDNA was added to 10 ml of 0.05 M Trizma buffer at PH 8 to give DNA concentrations ranging from 1 mg ml^{-1} to 1.5 mg ml^{-1} and stored at −18˚C until used. Four μl aliquots of ssDNA (at 1.5 mg ml^{-1}) were added to solutions of varying amounts of LH6 and cisplatin and the total volume was made up to 20 μl by adding mQ water so that the concentrations of compounds ranged from 1.87 to 60 μM. Sixteen μl of mQ water was added with 4 μl of ssDNA to prepare a DNA blank. Incubation was carried out in shaking water bath at 37˚C for 2 h following which 16 μl aliquots of drug-DNA mixtures were loaded onto the 1% agarose gel and electrophoresis was run in TAE buffer for 50 min at 120 V cm^{-1} at room temperature.

Table I. *Drug concentrations required for 50% cell kill (IC_{50}) and resistance factors (RF) of LH6, cisplatin (used as a reference compound), curcumin and quercetin applying to human ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cancer cell lines.*

	IC_{50} (μM)	IC_{50} (μM)			
Compound	A2780	A2780^{cisR}	RF	A2780^{ZD0473R}	RF#
LH6	1.61±0.05	1.20±0.01	0.75	2.22±0.15	1.37
Cisplatin	1.0±0.45	12.4±1.12	12.4	8.2±1.14	8.2
Curcumin	11.02±0.09	13.38±0.08	1.21	13.45±0.09	1.22
Quercetin	19.02±0.05	35.50±0.07	1.86	24.14±0.06	1.27

#Resistant factor (RF) is defined as the ratio of the IC_{50} value in the resistant cell line over that in the responsive cell line.

pBR322 plasmid DNA. pBR322 plasmid DNA was supplied in a 10 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer at a concentration of 0.5 μg/μl. Exactly 1 μl of supplied pBR322 plasmid DNA in solution was added to solutions of LH6 and cisplatin at different concentrations ranging from 1.87 to 60 μM.The total volume was made up to 20 μl by adding mQ water. The DNA blank was prepared by adding 19 μl of mQ water to 1 μl of pBR322 plasmid DNA. The samples were incubated for 5 h on a shaking water bath at 37˚C in the dark, at the end of which the reaction was quenched by rapid cooling to 0˚C for 30 min. The samples were thawed and mixed with 2 μl of marker dye (0.25% bromophenol blue and 40% of sucrose). Seventeen μl aliquots of drug-DNA mixtures containing 1 μl of DNA were loaded onto the 1% agarose gel made in TAE buffer (20) that contained ehidium bromide (1 mg ml^{-1}). Electrophoresis was carried out at room temperature for 1 h and 30 min at 150 V cm^{-1}.

BamH1 digestion. In this series of experiment, an identical set of drug-DNA mixtures, as that described previously, was first incubated for 5 h in a shaking water bath at 37˚C and then subjected to *BamH1* (10 units μl^{-1}) digestion. To each 20 μl of incubated drug-DNA mixture were added 3 μl of 10× digestion buffer SB followed by 0.2 μL *BamH1* (2 units).The mixtures were left in a shaking water bath at 37˚C for 1 h, at the end of which the reaction was terminated by rapid cooling. Electrophoresis was carried out for 1 h and 30 min at 150 V cm^{-1} and the gel was subsequently stained with ethidium bromide, visualized under UV light and photographed as described previously.

Cytotoxicity assay. The cell kill due to drugs alone and in combination were determined using the MTT (3-(4,5–Di-methyl-2-thiazole)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay (21, 22). Briefly, 4,000 to 5,500 cells (maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES, 0.112% sodium bicarbonate and 2 mM glutamine without

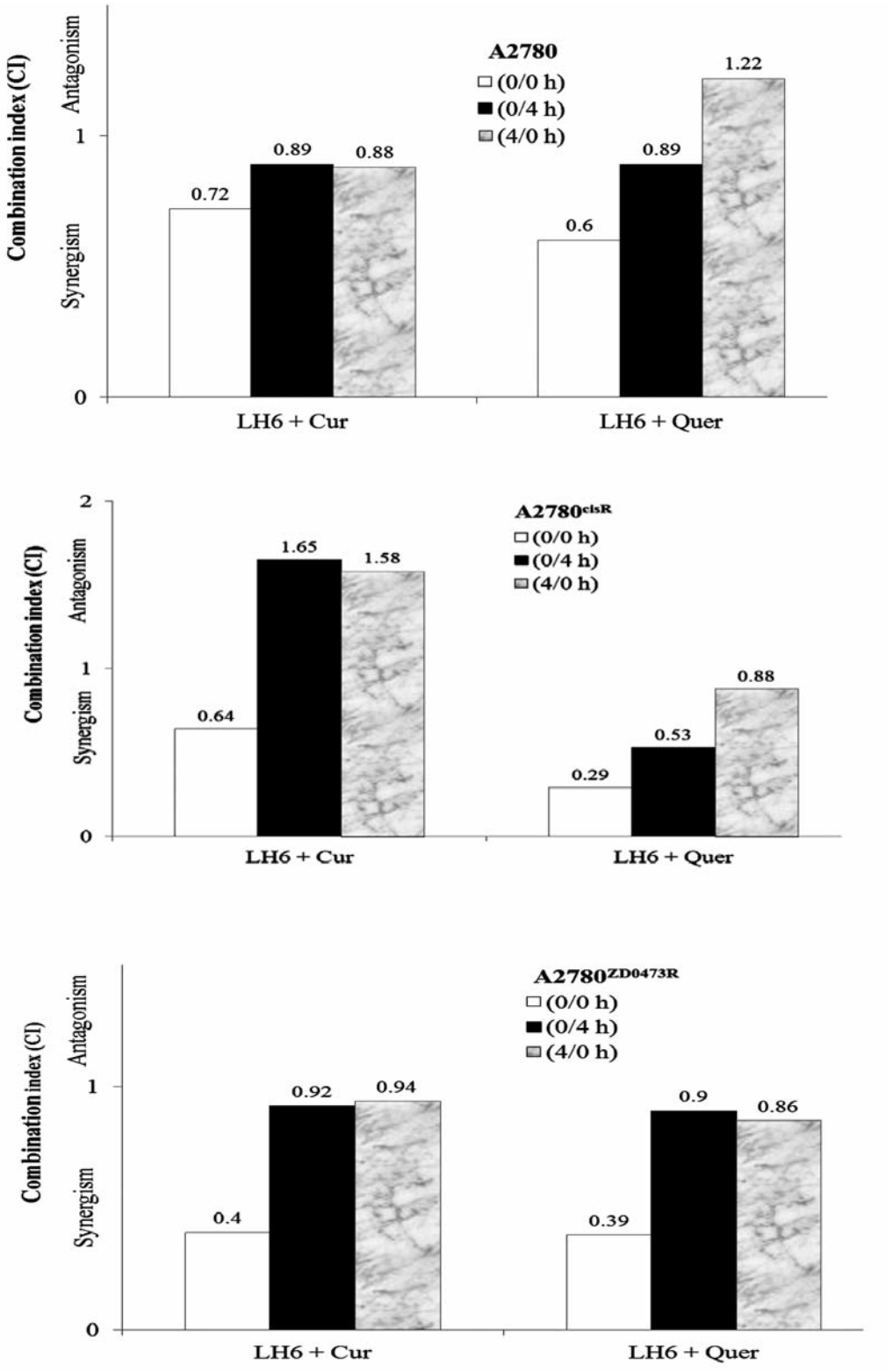

Figure 3. *Combination indices (CIs) applying to 0/0 h, 0/4 h and 4/0 h combinations of LH6 with curcumin (Cur) and quercetin (Quer) at the median effect dose (ED_{50}) in (a) A2780, (b) $A2780^{cisR}$ and (c) $A2780^{ZD0473R}$ cell lines, calculated based on the pooled data from three to five individual experiments; 0/0 h meant that both the compounds were administered at the same time, 0/4 h meant that LH6 was administered first followed by the phytochemical 4 h later and 4/0 h meant the converse.*

antibiotics) were seeded into flat-bottomed 96-well culture plates in 10% FBS/RPMI 1640 culture medium and allowed to attach overnight. For single-treatments, drugs were added at least three to five different concentrations to triplicate wells and left in an incubator (at 37˚C, with 5% carbon dioxide in air, at pH 7.4) for 72 h. Solutions of drugs made in 10% FCS/RPMI-1640 medium (0.16 to 20 μM for CS and LH4, 0.16 to 200 μM for quercetin and curcumin) and 100 μl of drugs were added to equal volumes of cell culture in triplicate wells, then left to incubate under normal growth conditions for 72 h at 37˚C in a humidified atmosphere. For combinations studies, cells were treated with increasing concentrations of drugs at constant ratios of their half maximal inhibitory concentration (IC_{50}) values concentrations, *i.e.* drug concentrations required for 50% cell kill, using the sequences: 0/0 h, 0/4 h and 4/0 h, where 0/0 h meant that both the drugs were added at the same time, 0/4 h meant that LH6 was added first followed by the addition of phytochemical (quercetin/curcumin) 4 h later and 4/0 h meant the converse. The concentration ranges were: LH6: 0.16-2.58 μM, 0.12-1.92 μM and 0.16-2.58 μM; Quercetin: 1.90-30.43 μM, 3.55-56.80 μM and 2.42-38.63 μM; Curcumin: 1.10-17.63 μM, 1.34-21.41 μM and 1.35-21.52 μM for A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines, respectively. At the completion of the 72 h incubation period, the MTT reduction assay was performed as described previously (21, 22).

Median effect analysis (23, 24), based on the pooled data from 3 to 5 individual experiments each comprising at least three data points for each drug alone and in combination, was carried out to calculate the combination index (CI) as a quantitative measure of combined action using the Calcusyn software (V2) (Biosoft, City, UK).

$$CI = \frac{D1}{D1\times} + \frac{D2}{D2\times},$$

where D1 and D2 in the numerator stand for the concentrations of compounds 1 and 2 in combination needed to achieve x% inhibition, whereas D1× and D2× in the denominator represent the same when they are present alone. In the following equation, D× denotes dose of drug, Dm is the median-effect dose, which is equivalent to IC_{50}, fa is the fraction of cells affected (*i.e.* killed) by the dose, fu is the fraction of cells remaining unaffected so that fu=1-fa and m is the exponent defining the shape of the dose effect curve.

$$D_x = D_m[f_a/(1-f_a)]^{1/m}$$

CI of <1, =1 and >1 indicates respectively synergism, additivity and antagonism in the combined drug action. The linear correlation coefficient 'r' was used as a measure of goodness of fit for the pooled data (where r=1 is a perfect fit). For the cell culture system, r should be greater than 0.95.

Cellular platinum accumulation and platinum-DNA binding. Exponentially growing A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cells in 10 ml 10 % FCS/RPMI 1640 culture medium (cell density: 1×10^6 cells ml^{-1}) were incubated with solutions of LH6 and cisplatin (at 25 μM for LH6 and 50 μM for cisplatin) for 2 h, 4 h and 24 h. Two sets of cell culture dishes were prepared; one set for DNA binding studies and the other for cellular accumulation of platinum. Cells were collected by scraper and the cell suspensions were transferred into 10 ml centrifuge tubes. The cell suspensions were spun at 3,500 rpm at 4˚C for 2 min to obtain the cell pellet. The supernatant was drained out and the cell pellets were washed with 4 ml PBS, which was kept in 4˚C. The mixtures were centrifuged again at 3,500 rpm at 4˚C for 2 min by using a CS-15R Centrifuge (Beckman, City, province, Australia) to obtain the cell pellet. After discarding the supernatant, 500 μl of the PBS was added to re-suspend the cells and the suspensions were transferred to corresponding labelled of 1.5 ml centrifugal Eppendorf tubes. Surviving cell concentration was determined by a TC10™ Automated Cell Counter (Bio-Rad, City, province, Australia) using trypan blue. The samples in the tubes were spun for 2 min at 10,000 rpm at 4˚C to obtain the pellet and stored at −20˚C until assayed for platinum contents. At least three independent experiments were performed for both cellular accumulation and DNA binding studies. Total platinum contents in cell pellets were determined by graphite furnace AAS.

Platinum-DNA binding. Following incubation of cells with drugs, high molecular weight DNA was isolated from cell pellet using the JETQUICK Blood DNA Spin Kit/50 (supplier, address) according to the modified protocol of Bowtell *et al*. (25). DNA content was determined by UV spectrophotometry (260 nm). Pt levels were determined by graphite furnace AAS. A_{260}/A_{280} ratios were found to be between 1.75 and 1.8 for all samples ensuring its high purity of the DNA (25) and the DNA concentration was calculated according to the following equation: Concentration=Absorbance at 260 nm×50 ng/μl.

Results and Discussion

Chemistry. Monofunctional platinum *tris*{imidazo(1,2α) pyridine} chloroplatinum(II) [coded as LH6] was synthesized according to modified Kauffman method (26). Cisplatin, used as a reference, was synthesized according to Dhara's method (27). Synthesis and characterization are fully described in the experimental section. Structures of LH6, curcumin, quercetin and cisplatin are shown in Figure 1.

Molar conductivity. Whereas non-polar and non-ionic substances may cross the cell membrane by both passive diffusion and carrier-mediated transport (28), polar molecules and ions usually cross the cell membrane by carrier mediated transports (28, 29). Cisplatin, being administered by intravenous route, will remain largely intact in the extracellular fluid that contains high chloride concentration. Hence, it is expected to cross the cell membrane by both passive diffusion and carrier-mediated transport (28). It also suggested that cisplatin can also cross the cell membrane by pinocytosis (30). Unlike cisplatin, designed monofunctional platinum compound LH6, being ionic in nature, is expected to cross the cell membrane by carrier-mediated transport only.

The limiting molar conductivity value (Λ_0) for LH6 was found to be 393 Ω^{-1} cm^2 mol^{-1} compared to 136 for cisplatin, reflecting the difference in nature of the compounds (ionic for LH6 and polar covalent for cisplatin) (31).

Drugs administered alone. Table I lists the IC_{50} values and the resistant factors (RF) of LH6, cisplatin (used as a reference), curcumin and quercetin applying to the ovarian cancer cell lines A2780 (cisplatin sensitive; the parent cell line), $A2780^{cisR}$ (cisplatin resistant) and $A2780^{ZDO473R}$ (picoplatin resistant) cell lines. IC_{50} represents the drug concentration required for 50% cell kill and RF is defined as the ratio of the IC_{50} value in the resistant cell line to that in the parent cell line.

Whereas the activity of cisplatin decreases markedly from the parent A2780 cell line to the resistant $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines, that of LH6 in $A2780^{cisR}$cell line remains the same as that in the parent A2780 cell line and shows less pronounced decrease in $A2780^{ZD0473R}$ cell line. The pronounced cytotoxicity of LH6 against all three ovarian cancer cell lines clearly indicates that it must differ from cisplatin in the mechanisms of anti-tumor action and the ability to overcome drug resistance. As LH6 contains three bulky imidazo(1,2-α)pyridine ligands compared to two ammonia ligands present in cisplatin, it will undergo distinctly different non-covalent interactions in terms of hydrogen bonding and stacking interactions with nucleobases in the DNA. These may translate into significant differences in protein recognition and, hence, the ability to induce cell death or the escape from it. Irrespective of the mechanisms involved, the results indicate that LH6 has been better able to overcome resistance operating in the ovarian cancer cell lines. In line with the results of the study, it has been reported that monofunctional cationic compounds containing bulky planaramine ligand can kill cancer cells with a greater efficacy than cisplatin and oxaliplatin (4, 31). Between the two phytochemicals, curcumin is found to be more active than quercetin against all three cell lines. Like LH6, the two phytochemicals have lower RF values than for cisplatin indicating the mechanisms of resistance operating in the resistant cell lines do not apply to the phytochemicals, as well as LH6.

Drugs in combination. Combinations of LH6 with curcumin and quercetin were administered to human ovarian A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cancer cell lines as a function of the sequence of administration and concentrations. Combination indices (CIs), median-effect dose (ED_{50}), dose required for 75% cell kill (ED_{75}) and that for 90% cell kill (ED_{90}), shape (sigmoidicity) and conformity (linear correlation coefficient r) are given in Table II. Figure 3 provides a bar graph of CI values at ED_{50}.

The results show that, for the combinations of LH6 with both Cur and Quer, at ED_{50} it was the concurrent administration that produced most synergistic outcome in all the three cell lines, whereas pre-treatment with phytochemical followed by the administration of LH6 4 hours later (4/0 h sequence of administration) was found to be least able to induce cell death (it should, however, be noted at ED_{90}, 4/0 h combination of LH6 with Cur but not with Quer produced most synergistic outcome as compared to two other sequences of administration). The findings are similar to those observed for the combinations of other monofuntional platinums and phytochemicals where bolus administration was found to be most synergistic (6), but different from those observed for the combinations of bifunctional platinums cisplatin and oxaliplatin with Cur and Quer where pre-treatment with the phytochemicals was found to produce most synergistic outcomes (32). The results indicate that the mechanisms underpinning synergistic outcome from the combination of Cur and Quer with monofunctional platinum LH6 are different than those for their combination with bifunctional platinums cisplatin and oxaliplatin (32). Like bifunctional platinums, monofunctional compounds with bulky planaramine ligands can also bind covalently to N7 position of guanine (but forming only monofunctionl adduct) but are expected to undergo stacking interaction and hydrogen bonding with DNA (4). But why at the median effect dose, co-administration of LH6 with Quer and Cur was found to be more synergistic than 0/4 h and 4/0 h sequences of administration remains an open question. Possible mechanisms involved in the combined drug are considered in the following paragraphs.

Further mechanisms of combined drug action. The results of the present study and the reported results (4-6) can be seen to indicate that the combined drug action from the monofunctional platinum and phytochemicals is a function of the nature of the phytochemical, that of the monofunctional platinum and status of the cell. Thus, although quercetin was found to be less cytotoxic than curcumin against both resistant $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines, in combination with LH6 it was more synergistic than curcumin at the median effect dose. This is not unexpected when we note that, although both the phytochemicals act as antioxidants and inducers of cell death (16), they differ in the mechanisms involved as described below. Thus, unlike curcumin, quercetin is known to increase the levels of intracellular ROS by producing quercetin radicals (Quercetin-O•) after peroxidase-catalyzed oxidation during scavenging reactive peroxyl radicals (17) so that quercetin can cause free radical-induced apoptosis through the ROS/AMPKα1/ASK1/p38 and the AMPKα1/COX2 signaling pathways (18). Quercetin radicals are also found to lower the intracellular GSH pool in a concentration-dependent manner (18) and to trigger apoptosis through mitochondrial de-polarization (19). It has been reported that quercetin can interact with type II estrogen binding site and was found to be synergistic with cisplatin in the killing of ovarian cancer cells, such as OVACA433, CAOV3 and SKOV3 (1). Also, the efficacy of doxorubicin, TRAIL and alkylating agents (DTIC and cisplatin) has been found to be enhanced by quercetin-

Table II. *Dose-effect parameters applying to combinations of LH6 with curcumin and quercetin in the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines; 0/0 h meant that both the compounds were administered at the same time, 0/4 h meant that LH6 was administered first followed by the phytochemical 4 h later and 4/0 h meant the converse.*

Drug	Sequence (h)	A2780			A2780^{cisR}			A2780^{ZD0473R}											
		CI at ED_{50}	CI at ED_{75}	CIs at ED_{90}	Dm	m	r	CI at ED_{50}	CI at ED_{75}	CIs at ED_{90}	Dm	m	r	CI at ED_{50}	CI at ED_{75}	CI at ED_{90}	Dm	m	r
LH6					1.62	1.61	0.99				1.23	1.67	0.99				1.58	1.68	0.99
Curcumin					9.51	1.10	0.99				11.3	1.43	0.99				1.96	1.22	0.97
LH6+	0/0	0.72	0.82	0.95	0.54	1.11	0.99	0.64	0.93	1.36	0.35	1.00	0.97	0.40	0.62	0.96	0.29	0.89	0.97
Curcumin	0/4	0.89	0.86	0.87	0.66	1.31	0.99	1.65	1.71	1.77	0.92	1.47	0.98	0.92	0.62	0.91	0.68	1.40	0.99
	4/0	0.88	0.75	0.66	0.67	1.58	0.99	1.58	1.64	1.72	0.88	1.45	0.97	0.94	0.90	0.87	0.70	1.46	0.99
Quercetin					28.52	1.56	0.99				24.72	1.14	0.98				1.77	1.87	0.98
LH6+	0/0	0.60	0.91	1.37	0.58	0.98	0.99	0.29	0.55	1.06	0.16	0.78	0.96	0.39	0.66	1.15	0.31	0.85	0.99
Quercetin	0/4	0.89	1.03	1.19	0.85	1.28	0.99	0.88	1.06	1.35	0.49	1.11	0.99	0.90	0.95	1.03	0.73	1.36	0.99
	4/0	1.22	1.42	1.65	1.17	1.28	0.94	0.53	0.75	1.09	0.29	0.97	0.94	0.86	1.02	1.24	0.70	1.19	0.99

Table III: *Average binding ratio of platinum to DNA in A2780 and A2780^{cisR} cells after their interaction with solutions of cisplatin and LH6 (at 50 μM in the case of cisplatin and 10 μM in the case of LH6) for 2, 4 and 24 h at 37˚C.*

Drugs	Average binding ratio [Pt(nmol)/DNA (nmol)]×103 in:					
	A2780			A2780^{cisR}		
	2 h	4 h	24 h	2 h	4 h	24 h
Cisplatin	0.38±0.02	0.69±0.02	2.50±0.06	0.78±0.06	0.73±0.05	1.54±0.28
LH6	0.70±0.04	0.68±0.06	0.44±0.01	1.45±0.10	0.84±0.04	0.59±0.06

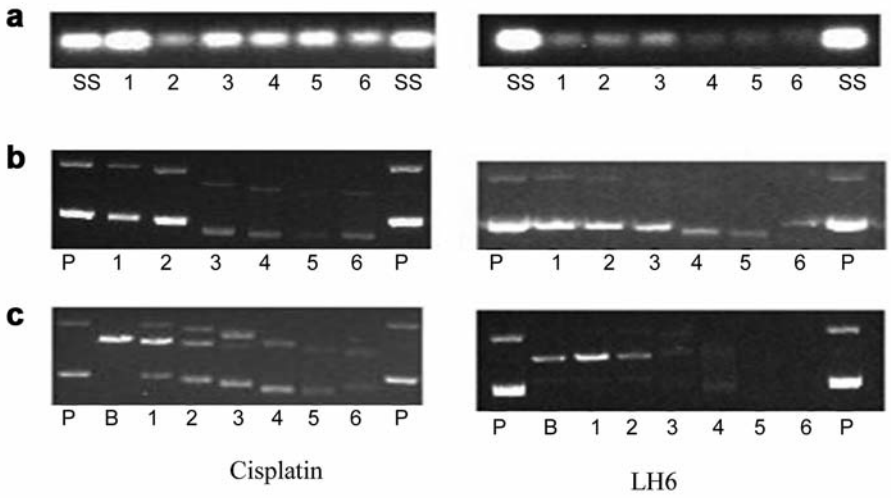

Figure 4. *Electrophoretograms applying to the interaction of a: salmon sperm DNA (ssDNA); b: pBR322 plasmid DNA without BamH1 digestion; c: pBR322 plasmid DNA followed by BamH1 digestion, with increasing concentrations of cisplatin and LH6. Lanes: SS: Untreated salmon sperm DNA; P: untreated pBR322 plasmid DNA; B: untreated and digested pBR322 DNA by BamH1 digestion; 1: 1.87 μM; 2: 3.75 μM; 3: 7.5 μM; 4: 15 μM; 5: 30 μM; 6: 60 μM.*

mediated chemosensitization (15, 33). This is believed to be associated with its ability to down-regulate HIF1-a, HER2/neu and/or due to persistent T-cell tumor-specific responses. Both *in vitro* and *in vivo* studies have shown that quercetin-mediated down-regulation of HIF1-a, HER2/neu and induction of persistent T-cell tumor-specific responses in cancer cells makes them sensitive to doxorubicin, sparing normal cells (34). The ability of quercetin to redistribute TRAIL receptors and other components of death-inducing signalling complex (DISC) into lipid rafts is considered to sensitize colon cancer cells to TRAIL-mediated apoptosis. It is believed to be one of the major mechanisms of its chemosensitizing efficacy (35). DTIC, an alkylating agent approved by the FDA for the treatment of melanoma (36), on its own has only moderate response rates (37). Pre-treatment with quercetin was found to promote an ataxia-telangiectasia mutated (ATM)-dependent phosphorylation of p53 (38) and induce its transcriptional activity (39), thereby sensitizing melanoma cells to DTIC. A number of studies have shown that quercetin is highly effective as a potent and non-toxic drug that can cause the reversal of multidrug resistance (MDR) in several tumor models (40). It can also sensitize HeLa cells to cisplatin-induced apoptosis through the down-regulation of heat shock protein (Hsp72) and multidrug resistance protein (MRP) (41). The synergistic effect of quercetin with cisplatin could also be mediated through the down-regulation of Bcl-2 and Bcl-xL with concomitant up-regulation of Bax and the induction of mitochondrial membrane permeabilization, as observed in Hep-2 cells (42). The results of the present study indicate that quercetin may be a promising candidate for combination with tumor active monofunctional platinums towards overcoming cisplatin resistance in ovarian cancer.

The second phytochemical curcumin, as noted earlier, is a polyphenol and a key component of turmeric that has antioxidant, anti-toxic, anti-inflammatory, cancer chemo-

preventive and chemotherapeutic properties (1, 8). It has been suggested that both the platinum resistance and the enhancement of platinum action, due to its combination with phytochemicals, may be related to the multidrug resistance gene *NF-κB*. Whereas resistance to platinum drugs may be associated with aberrant activation of NF-κB, the synergistic combination of LH6 and curcumin is thought to have dampened its expression. In addition to NF-κB, other mediators are also believed to be involved in platinum based chemotherapy (43). For example, curcumin has been found to sensitize ovarian cancer cells (CAOV3 and SKOV3) to cisplatin-induced apoptosis (12, 13) by inhibiting the FA/BRCA pathway and the production of cytokine interleukin 6 (IL-6) (1). Like quercetin, curcumin may also be a promising candidate for combination with monofunctional platinum.

Interaction with DNA. Figure 4 gives the electrophoretograms applying to the interaction of (a) ssDNA, (b) pBR322 plasmid DNA without BamH1 digestion and (c) pBR322 plasmid DNA followed by BamH1 digestion, with increasing concentrations of cisplatin and LH6 ranging from 1.87 to 60 μM (lane 1 to 6) for 5 h at 37˚C. Lane P applied to the untreated pBR322 plasmid DNA that served as control.

In case of ssDNA, only a single band was observed in both untreated and treated ssDNA. There was a decrease in intensity of the band at higher concentrations of LH6 indicating that the compound was able to induce DNA damage at higher concentrations. In contrast, no noticeable change in band intensity in the case of cisplatin indicates that cisplatin was unable to cause any damage to ssDNA in the studied range of concentration. As noted earlier, LH6 can only form monofunctional adducts with DNA as against predominantly intrastrand 1,2-Pt(GG) and 1,2-Pt(AG) adducts formed by cisplatin (although cisplatin is also known to form monofunctional adducts and less frequent but more cytotoxic interstrand adducts with DNA) (44) that cause significant change in DNA conformation at and close to the binding site (45). However, this was not reflected as any observable change in mobility of the linear ssDNA band. Damage to ssDNA at higher concentrations of LH6 is attributable to non-covalent interactions involving imidazo(1,2-α)pyridine ligand, such as stacking interaction and hydrogen bonding.

When pBR322 plasmid DNA was interacted with increasing concentrations of LH6, there was a decrease in intensity of DNA bands (at higher concentrations) and a change in mobility (especially for form I) indicating change in DNA conformation. This may be attributed to the presence of the bulky imidazo(1,2-α)pyridine ligand. When pBR322 plasmid DNA was interacted with cisplatin, two bands corresponding to forms I and II were observed at all concentrations of the compound ranging from 0 to 60 μM. However, there was a decrease in separation between the bands and a decrease in intensity of the bands with the increase in concentration of cisplatin. The results are indicative of conformational change in the DNA and the occurrence of DNA damage. As noted earlier, cisplatin forms mainly intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts with duplex DNA that causes significant bending of the DNA strand at and close to the binding site, thus bringing about changes in conformation especially to the supercoiled form I DNA (44).

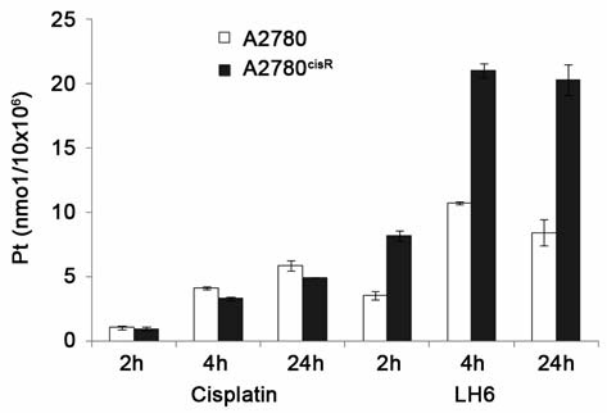

Figure 5. *Levels of platinum accumulation in A2780 and A2780^{cisR} cells after their interaction with solutions of LH6 and cisplatin (50 μM in case of cisplatin and 10 μM in case of LH6) for 2, 4 and 24 h at 37˚C.*

Cellular accumulation of Pt as a function of time. To explore the idea that whereas cisplatin can cross the cell membrane by both passive diffusion and carrier-mediated transport (46, 47) monofunctional platinum compounds do so only by carrier-mediated transport (31), cellular accumulations of platinum from cisplatin and monofunctional platinum LH6 were determined as a function of duration of incubation.

Figure 5 gives the changes in Pt accumulation in A2780 and A2780^{cisR} cell lines as a function of time as applied to cisplatin and LH6 at 2, 4 and 24 h (at 50 μM concentration for cisplatin and 10 μM for LH6). At 24 h, LH6 is found to have lower Pt accumulations than cisplatin in both the cell lines. However, at 2 h and 4 h the values for LH6 are higher than cisplatin in both the resistant cell lines. Whereas Pt accumulation from cisplatin is found to increase almost linearly with time, for LH6 it reaches maximum at 4 h and is somewhat lower at 24 h. As noted earlier, whereas cisplatin is expected to cross the cell membrane by both passive diffusion and carrier-mediated transport (48), LH6 (being ionic in nature) can cross the cell membrane only by carrier-mediated transport so that their total flux of transport into the cell can be lower (31). However, LH6 is found to result in higher Pt accumulation than cisplatin at all time points especially in the resistant cell line. It is possible that the presence of bulky hydrophobic imidazo(1,2-α-pyridine) ligand in LH6 facilitates its uptake through the cytoplasmic membrane and it is also

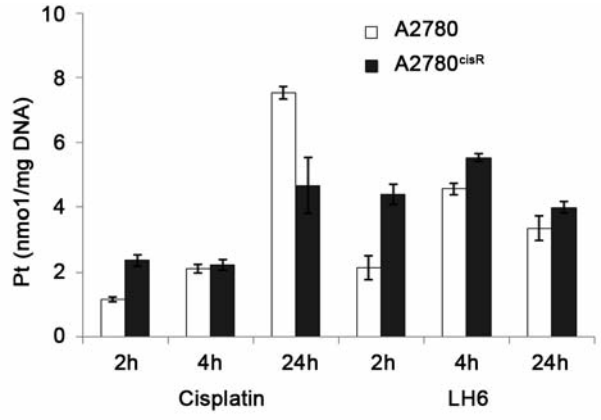

Figure 6. *Pt-DNA binding A2780 and A2780^{cisR} cells after their interaction with solutions of cisplatin and LH6 (50 μM in case of cisplatin and 10 μM in case of LH6) for 2, 4 and 24 h at 37˚C.*

possible that LH6 is better able to bind to the carrier molecules so that it is more efficiently transported. Somewhat lower Pt accumulation at 24 h than at 4 h for LH6 can be seen to imply that efflux processes may become more dominant as the period of incubation was increased.

Platinum-DNA binding as a function of time. Platinum-DNA binding. Figure 6 gives Pt-DNA binding levels (nmol Pt per mg DNA) in A2780 and A2780^{cisR} cells after their interaction with solutions of compounds (50 μM for cisplatin and 10 μM for LH6) for 2 h, 4 h and 24 h. Pt-DNA binding level is found to increase with time in the case of cisplatin and reach maxima at 4 h in the case of LH6. At 24 h, cisplatin has the highest Pt-DNA binding level in the parent A2780 cell line, whereas LH6 has the higher Pt-DNA binding levels at 2 h and 4 h in both the parent and resistant cell lines than at 24 h. As noted earlier, LH6, being positively charged, would be readily attracted to the negatively charged DNA, thus undergoing initially associative interaction followed by covalent binding (31). It has been reported that pyridine ligand in monofunctional platinums can sterically clash with base pairs of the double helix, in effect twisting the bases out of their native conformation. This may explain why monfunctional platinum can inhibit transcription (49).

Platinum-DNA binding ratio (r). The Pt-DNA binding ratio was calculated as a function of time to determine how the ratio changed as the period of incubation was increased.

Table III gives the average binding ratio of platinum to DNA in A2780 and A2780^{cisR} cells after their interaction with solutions of cisplatin and LH6 (at 50 μM in case of cisplatin and 10 μM in the case of LH6) for 2, 4 and 24 h at 37˚C. The values were calculated based on the average molecular mass of 330 for a nucleotide (660 for a pair) in the DNA.

When Pt-DNA binding ratios at 24 h are compared, cisplatin is found to have resulted in higher binding ratio in the parent A2780 cell line, whereas for LH6 the value was greater in the resistant cell line. The values can be seen to be in line with greater activity of cisplatin in the parent cell line and that of LH6 in the resistant cell line. The other point to note is that, whereas for cisplatin the binding ratio is greatest at 24 h, for LH6 the value is highest at 2 h. A further point is that less than one percent of purine nucleotides was found to be saturated with platinum even at the maximum binding level, possibly because DNA damage and cell death may eventuate before any higher levels can be achieved. The results may also be seen to illustrate sequence specificity of platinum-DNA binding, even for monofunctional platinum LH6. As the number of GG sites in DNA are found to be lower than the numbers of G and A sites (a tree diagram can show that number of GG sites would be 1 out of 16, whereas that of G and A sites together would be 2 out of 4 or 8 out of 16) (50), a lower Pt-DNA binding ratio is expected to result from cisplatin at 2 h and 4 h than from LH6 for both the cancer cell lines.

Conclusion

Newly-synthesized monofunctional platinum coded as LH6 is found to be significantly more active against the resistant A2780^{cisR} cells than the parent A2780 cell line, indicating that it has been able to overcome platinum resistance. The observed synergism from combinations of LH6 with curcumin and quercetin strengthens its ability to induce cell death and provides support to the idea that the synergistic combinations of targeted-therapy and tumor active phytochemicals may provide an effective and affordable means of overcoming platinum resistance in ovarian cancer.

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