Combination of Genistein and Cisplatin with Two Designed Monofunctional Platinum Agents in Human Ovarian Tumour Models

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Abstract. *A great amount of research effort has been directed at platinum compounds that bind with DNA differently from cisplatin with the idea that the difference may translate into an altered spectrum of activity. Recently research has also been directed at applying combinations of platinum agents with tumour-active phytochemicals with the aim of providing a means of overcoming platinum resistance in ovarian cancer. Herein we report the synthesis of monofunctional platinum tris(3-hydroxypyridine)chloroplatinum(II) chloride (coded as LH1) and tris(imidazole)chloroplatinum(II) chloride (coded as LH2), and their activity alone and in combination with genistein and cisplatin against human ovarian A2780, cisplatin-resistant A2780^{cisR} and picoplatin-resistant A2780^{ZD0473R} cancer cell lines. Although both LH1 and LH2 were found to be less active than cisplatin against the tumour models, they produced synergistic outcomes in combination with genistein. Both the level of cellular accumulation of Pt and of Pt–DNA binding resulting from the combination were greater in the A2780^{cisR} cell line than in the parental A2780 cell line, irrespective of the sequence of administration. Absence of association between activity of LH1 and LH2 and the level of Pt–DNA binding indicates that the cell death induced by LH1 and LH2 may not be limited to the effect of their binding with DNA.*

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Key Words: Ovarian cancer, 3-hydroxypyridine, imidazole, DNA fragmentation, drug resistance, drug combination.

Although ovarian cancer generally responds well to the combination of platinum drugs (cisplatin or carboplatin) with paclitaxel, these drugs often fail to function when relapse occurs (1). Whereas commonly used platinum drugs bind with DNA to form mainly intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts, monofunctional cationic platinum compounds (as the name implies) can only form monofunctional adducts with DNA (2-5). As a part of our ongoing effort to design novel planaramine-containing platinum-based anticancer agents (6), herein we report the synthesis, characterization, and activity against human ovarian cancer cell lines of two monofunctional platinum compounds namely tris(3-hydroxypyridine)chloroplatinum(II) chloride (coded as LH1) and tris(imidazole)chloroplatinum(II) chloride (coded as LH2). The cell lines A2780, and its cisplatin-resistant (A2780^{cisR}) and ZD0473-resistant (A2780^{ZD0473R}) derived lines were obtained from Dr. Philip Beale (NSW Cancer Centre, Concord Hospital, Australia). Initially, the parental A2780 cell line derived from fresh tissue of a patients with ovarian cancer who had not been treated. The parental cell line was exposed to increasing concentrations of cisplatin intermittently until it became resistant to cisplatin, thus producing the A2780^{cisR} cell line. Practically the same procedure was employed to produce A2780^{ZD0473R} except that the parental cell line was exposed to increasing concentrations of the platinum drug ZD0473 (7). Once developed, resistance was found to be maintained as observed by other researchers (8), no significant change in the half-maximal inhibitory concentration (IC_{50}) value for cisplatin (taken as a reference compound) against the cell lines being taken as the criterion.

The interactions of LH1 and LH2 as well as cisplatin with pBR322 plasmid DNA with and without *BamH*I digestion and damage to cellular DNA were also investigated. We also investigated the effect of sequenced combinations of LH1 and LH2 with cisplatin and the phytochemical genistein on cell death. Genistein (Figure 1) is a soy bean-derived

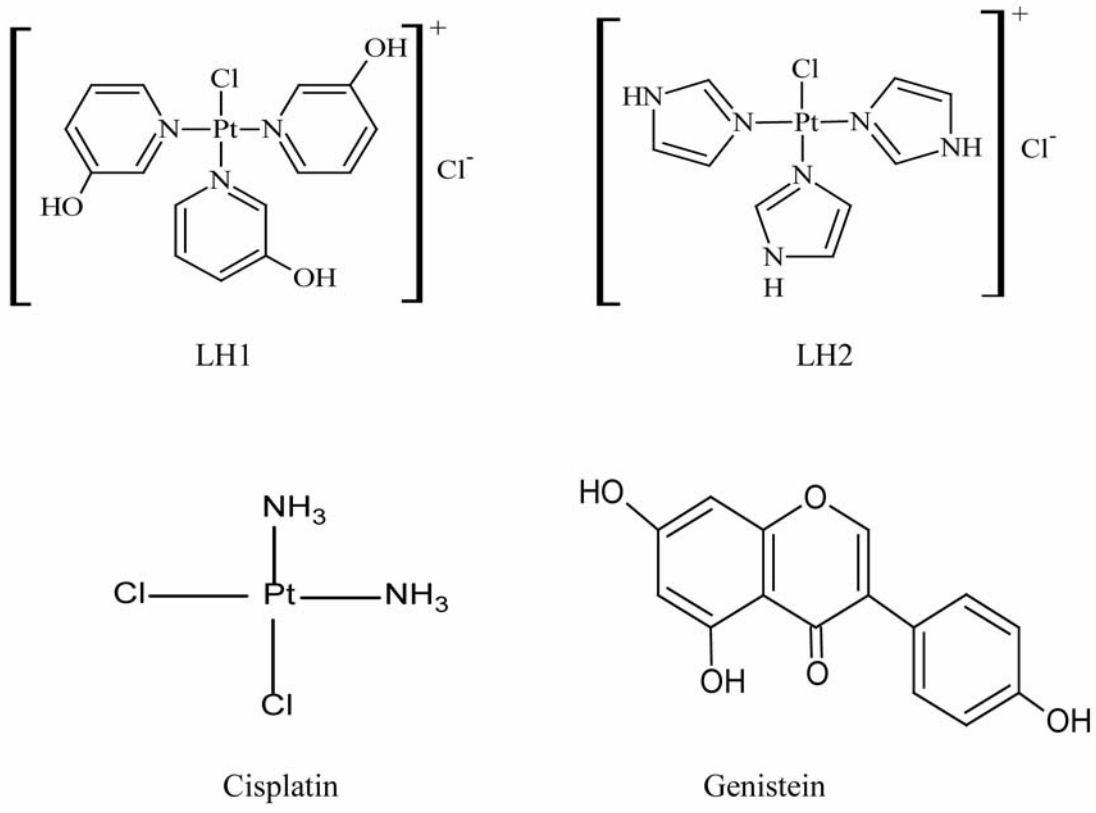

Figure 1. *Structures of LH1, LH2, cisplatin and genistein.*

isoflavone best known for its ability to inhibit cancer progression. Genistein is structurally similar to oestrogen but has more potent biological activity (9, 10). It acts as an inhibitor of protein tyrosine kinase, thus attenuating the growth of cancer cells (11-13). Genistein can suppress tumour necrosis factor (TNF)-induced activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), nuclear translocation of p65 and subsequent gene expression. It also inhibits the protein kinase B (AKT) pathway, thus enhancing necrotic-like cancer cell death (14). Recent studies have shown that genistein can suppress growth of gynaecological carcinoma (15). In contrast, two important factors responsible for platinum resistance are related to increased expression of NF-κB and serine/threonine protein AKT signalling that can lead to cell proliferation, anti-apoptosis, angiogenesis and metastasis. Hence, it is thought that combinations of platinum drugs with genistein can produce synergistic outcomes in ovarian tumour models (16). The structures of LH1, LH2, cisplatin and genistein are given in Figure 1.

Materials and Methods

Materials. Potassium tetrachloroplatinate (K_2PtCl_4), *N,N*-dimethylformamide (DMF), 3-hydroxypyridine and imidazole were from Sigma Chemical Company (St. Louis, MO, USA); hydrochloric acid (HCl) was from Asia Pacific Specialty Chemicals Ltd. (Auburn, NSW, Australia); ethanol was from Merck Pty. Ltd. (Kilsyth, Australia); pBR322 plasmid DNA was from ICN Biomedicals (Aurora, OH, USA); fetal calf serum, 5xRMPI 1640, 200 mM L-glutamine and 5.6% sodium bicarbonate (Trace Biosciences Pty. Ltd. (Castle Hill, NSW, Australia) JETQUICK Blood DNA Spin Kit/50 was from Astral Scientific (Caringbah, NSW, Australia).

Synthesis of LH1 and LH2. LH1 and LH2 were synthesized from potassium tetrachloroplatinate according to the modified Kauffman method (17). Briefly, K_2PtCl_4 (1 mmol, 0.415 g) dissolved in milli-Q (mQ) water (7.5 ml) was treated with concentrated hydrochloric acid (0.25 ml) followed by the addition at 50˚C of 10 mmol of 3-hydroxypyridine (0.9510 g) in the case of LH1 and imidazole (0.6809 g) in the case of LH2, dissolved in a mixture of 2 ml of DMF and 2 ml of mQ water. After stirring for 1 h, the volume was reduced to around 5 ml, followed by the addition of 30 ml of 6 M hydrochloric acid. The temperature was reduced to 30˚C and stirring was continued for 24 h until the volume was reduced to about 4 ml.

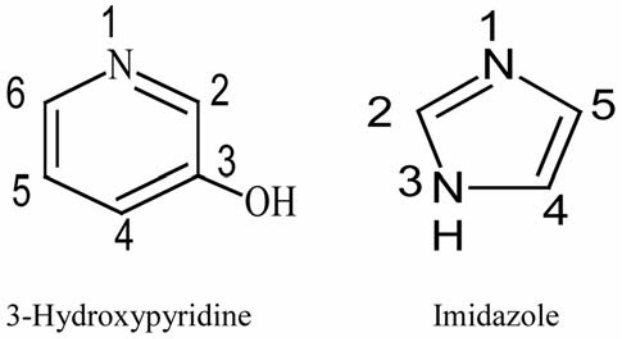

Figure 2. *Numbering scheme adopted for 3-hydroxypyridine and imidazole.*

The mixture was cooled by placing in an ice bath. The precipitate (LH1 and LH2) was collected by filtration, washed with ice-cold water–ethanol mixture and air-dried. The volume of the filtrate was reduced to about 4 ml to obtain further crop of LH1 and LH2. The crude products were purified by re-precipitation from 0.100 M HCl. Cisplatin used as a reference compound was synthesized according to Dhara's method (18).

Molar conductivity. The limiting molar conductivity values of the solutions of cisplatin, LH1 and LH2, first dissolved in 1:4 mixture of DMF and mQ water followed by further dilution with mQ water, were determined to ascertain the nature of the compounds (19).

Characterization. C, H, and N were determined using a Carlo Erba 1106 automatic analyzer available at the Australian National University Canberra ACT Australia whereas Pt was determined by graphite furnace atomic absorption spectroscopy (AAS). As LH1 and LH2 could not be obtained in crystalline form, infrared (IR), mass spectroscopy (MS) and 1H nuclear magnetic resonance (NMR) spectra were used to aid in their structural characterization. The IR spectra were obtained using a Varian FT-IR spectrometer (Bruker IFS66 spectrometer). To obtain mass spectra, solutions of LH1 and LH2 in 90% methanol and 10% DMF were sprayed into a Finnigan LCQ mass spectrometer. To obtain 1H NMR spectra using a Bruker DPX400 spectrometer at 400.2 MHz, compounds were dissolved in deuterated dimethyl sulfoxide (DMSO) and prepared in 5 mm high-precision Wilmad NMR tubes. In 1H NMR, s, d and q denote singlet, doublet and quartet, respectively. The numbering scheme adopted for 3-hydroxy pyridine and imidazole is given in Figure 2.

LH1: Pale yellow powder (445 mg, 86%); ^{1}H NMR (400MHz, [D_6] DMSO): δ (ppm)=8.85 (s, due to OH), 8.72 (s, due to C_2H), 7.44 (d, due to C_4H), 7.33 (q, due to C_5H), 3.40 (s, due to water), 2.50 (s, due to DMSO); IR (KBr): 3280, 2359, 1582, 1444, 1281, 1219, 1109, 1028, 809, 697, 580, 502 cm^{-1}; MS (ESI) *m/z* (%): 287.47 (100)=[$C_{15}H_{15}ClN_3O_3Pt$ – C_5H_5NO – 2Cl – H], 515.87 (90)=[$C_{15}H_{15}ClN_3O_3Pt$ – Cl], 514.40 (72)=[$C_{15}H_{15}ClN_3O_3Pt$– Cl – H], 323.27 (35)=$C_{15}H_{15}ClN_3O_3Pt$ – $(C_5H_5NO)_2$ – Cl – 2H]; Anal. calcd for $C_{15}H_{15}ClN_3O_3Pt$: C 32.6, H 2.7, N 7.6, Pt 35.4, found: C 32.4±0.4, H 2.7±0.4, N 7.3±0.4, Pt 35.4±1.0.

LH2: As a pale yellow powder (361 mg, 76%); ^{1}H NMR (400 MHz, [D_6] DMSO: δ (ppm)=7.76 (s, due to NH), 7.56 (s, due to C_2H), 6.92 (d, due to C_5H *meta*), 4.60 (s, due to water), 2.49 (s, due to DMSO); IR (KBr): 3250, 3145, 2354, 2341, 1700, 1650, 1550, 1512, 1427, 1200, 1140, 833, 779, 665, 609 cm^{-1}; MS (ESI) *m/z* (%): 465.73 (100)=[$C_9H_{12}Cl_2N_6Pt$ – 4H], 329.80 (41)=[$C_9H_{12}Cl_2N_6Pt$ – $(C_3H_4N_2)_2$ – 4H], 233.20 (98)=[$C_9H_{12}Cl_2N_6Pt$ – $(C_3H_4N_2)_3$ – Cl]; Anal. calcd for $C_9H_{12}Cl_2N_6Pt$: C 22.99, H 2.6, N 17.9, Pt 41.5, found: C 22.68±0.4, H 2.7±0.4, N 17.6±0.4, Pt 41.6±1.0.

Interaction with DNA. Interaction of LH1 and LH2 with pBR322 plasmid DNA (with and without *BamH*I digestion) was 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 from UVItec Limited (Cambridge, UK) was used for taking images of gels. Images were analyzed using Kodak molecular imaging software (Kodak MI software). Exactly 1 μl of supplied pBR322 plasmid DNA in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer was added to solutions of LH1, LH2 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) then 17 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 ethidium bromide (1 mg ml^{-1}). An identical set of drug–DNA mixtures was first incubated for 5 h in a shaking water bath at 37˚C and then subjected to BamHI (10 units μl^{-1}) digestion. To each 20 μl of incubated drug–DNA mixture were added 3 μl of 10x digestion buffer SB followed by 0.2 μl BamHI (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.

Cytotoxicity assay. Cell death due to incubation with drugs alone and in combination for 72 h was determined using the (3-(4,5-dimethyl-2-thiazole)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay (21, 22). The drug concentrations required for 50% cell kill (IC_{50}) were obtained from the results of quadruplicate determinations of at least three independent experiments. For combination studies, cells were treated with increasing concentrations of drugs at equipotent ratio using the sequences: 0/0 h, 0/4 h and 4/0 h, where 0/0 h means that both drugs were added at the same time, 0/4 h that genistein or cisplatin was added first followed by the addition of LH1 or LH2 4 h later, and 4/0 h meant the converse. The concentration ranges were: for A2780 cells: cisplatin: 0.10-1.59 μM, genistein: 1.50-24.03 μM, LH1: 5.02-80.29 μM and LH2: 2.65-42.35 μM; for A2780^{cisR} cells: cisplatin: 1.48-23.69 μM, genistein: 2.78-44.48 μM, LH1: 6.43-102.77 μM and LH2: 3.55-56.72 μM; and for A2780^{ZD0473R} cells: cisplatin: 1.40-22.32 μM, genistein: 1.63-25.99 μM, LH1: 6.34-101.49 μM and LH2: 3.81-60.88 μM.

Median-effect analysis was carried-out to calculate the combination index (CI) as a quantitative measure of combined action. This was based on the pooled data from three to five individual experiments each comprising at least three data points for

each drug and for each combination. The CI for two drugs in combination was calculated using the following equation (23) and using Calcusyn software (V2)(Biosoft, Cambridge, UK).

$$CI = \frac{D_1}{D_{1x}} + \frac{D_2}{D_{2x}}$$

where D_1 and D_2 are the concentrations of compounds 1 and 2 in combination needed to achieve x% inhibition, whereas D_{1x} and D_{2x} represent the same when used alone. In the following equation, D_x denotes the dose of drug, D_m is the median-effect dose which is equivalent to the IC_{50}, f_a is the fraction of cells affected (*i.e.* killed) by the dose, f_u is the fraction of cells remaining unaffected so that $f_u=1-f_a$ and m is the exponent defining the shape of the dose–effect curve.

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

CIs of <1, 1 and >1 indicates synergism, additivity and antagonism, respectively, 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.

Platinum accumulation and Pt–DNA binding. A2780, A2780^{cisR} and A2780^{ZD0473R} cells in culture medium were incubated with solutions of LH1, LH2 and cisplatin (at 50 μM) 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 2600 × *g* 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 phosphate-buffered saline (PBS) kept at 4˚C. The mixtures were centrifuged again at 2600 × *g* at 4˚C for 2 min by using CS-15R Centrifuge (Beckman, Palo Alto, CA 94304, USA) to obtain the cell pellet. After discarding the supernatant, 500 μl of PBS was added to re-suspend the cells and the suspensions were transferred to correspondingly labelled 1.5 ml centrifugal eppendorf tubes. Surviving cells were determined by TC10™ Automated Cell Courter (Bio-Rad, Gladesville NSW, Australia) using tryphan blue. The samples in the tubes were spun for 2 min at 7500 ×g at 4˚C to obtain the pellet and stored at −20˚C until assayed for platinum content. At least three independent experiments were performed for both the cellular accumulation and DNA-binding studies.

Total platinum content in cell pellets were determined by graphite furnace AAS. The method used for the determination of total intracellular platinum and Pt–DNA level was a modification of that described by Di Blasi *et al*. (24).

DNA fragmentation. DNA isolated from A2780 and A2780^{cisR} cells after incubation with combinations of genistein and cisplatin with LH1 and LH2 for 24 h using 0/0 h, 0/4 h and 4/0 h sequences of administration, according to the modified protocol of Bowtell (25) (as described previously), were subjected to agarose gel (2%) electrophoresis containing ethidium bromide (again as previously). The amount of the DNA was kept constant (at 0.5 μg). At the end of the electrophoresis, the gel was stained in the same TAE buffer (20) at room temperature for 1 h and 30 min at 150 V cm^{-1}. DNA bands were viewed under UV light and photographed as described previously.

Results and Discussion

Synthesis. LH1 and LH2 were synthesized and characterised based on microanalysis, spectral studies (IR, ^{1}HNMR and M and molar conductivity measurements. The limiting molar conductivity values (Λ_0) for cisplatin, LH1 and LH2 were 136, 184 and 248 Ω^{-1} cm^2 mol^{-1} respectively. Whereas cisplatin is expected to cross the cell membrane by both passive diffusion and carrier-mediated transport (26) reportedly also by pinocytosis (27), LH1 and LH2 are likely to cross the cell membrane by carrier-mediated transport only (4). Experiments on cellular accumulation of platinum as a function of concentration were carried-out to provide further information on this matter.

Interaction with DNA.

pBR322 plasmid DNA: Figure 3a shows the electrophoretograms for the interaction of pBR322 plasmid DNA for 5 h at 37˚C with increasing concentrations of LH1, LH2 and cisplatin ranging from 0 to 60 μM and Figure 3b gives the same applying to the interaction of pBR322 plasmid DNA for 5 h at 37˚C with increasing concentrations of compounds followed by *BamH*I digestion for a period of 1 h at 37˚C. At higher concentrations of LH1, separation between forms I and II bands decreased coupled and there was a significant decrease in intensity of the bands at and above 60 μM that was indicative of DNA damage. The change in mobility of DNA bands confirms that monofunctional adducts formed by LH1 were able to induce changes in DNA conformation (28). No observable change in either mobility or intensity of the pBR322 plasmid DNA bands in the case of LH2 indicates that the compound was not able to cause observable changes in the DNA. When pBR322 plasmid DNA interacted with cisplatin, two bands corresponding to forms I and II were observed at all concentrations ranging from 0 to 60 μM but there was a decrease in band separation and intensity of the bands with increasing concentration. The results are indicative of conformational change in the DNA and the occurrence of DNA damage.

BamHI digestion: Untreated and undigested pBR322 plasmid DNA gave two bands corresponding to forms I and II, while untreated but *BamH*I-digested pBR322 plasmid DNA gave only the form III band (29). The presence of forms I, II and III bands at low concentrations of LH1 (1.87 μM to 15 μM), I and II bands at 30 μM, and only one coalesced (consisting of forms I and II) band at 60 μM, is indicative of changes in DNA conformation. The presence of forms I, II and III bands at all concentrations of LH2 ranging from 1.87 μM to 60 μM indicates the compound was much less able to induce conformational change in pBR322 plasmid DNA. The presence of forms I, II and III bands at low concentrations of cisplatin (1.87 μM to 7.5 μM) and forms I and II bands at higher

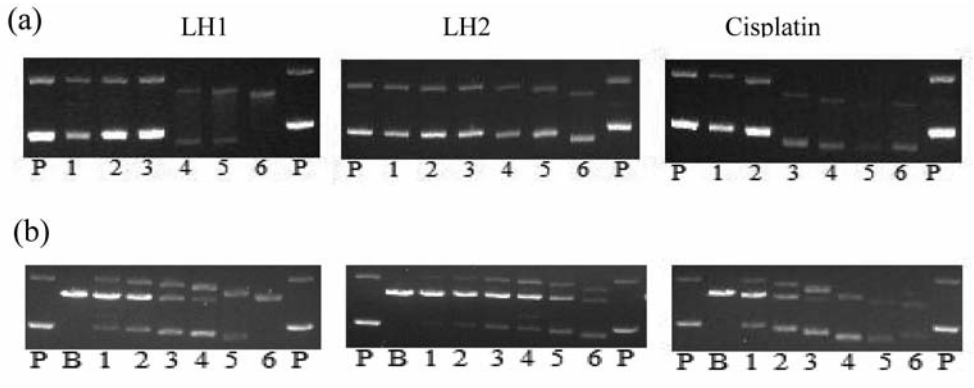

Figure 3. *Electrophoretograms for the interaction of pBR322 plasmid DNA with increasing concentrations of LH1, LH2 and cisplatin without (a) and with (b) BamHI digestion. P: Untreated pBR322DNA; B: untreated but BamHI-digested pBR322 DNA; lanes 1-6: pBR322 plasmid DNA interacted with increasing concentrations of compounds (1: 1.87 μM; 2: 3.75 μM; 3: 7.5 μM; 4: 15 μM; 5: 30 μM; 6: 60 μM).*

Table I. *The mean drug concentration (μM) required for 50% cell kill (IC_{50}) and resistance factors (RF) for LH1, LH2, cisplatin and genistein against the human ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}‡.*

Drugs	A2780	A2780^{cisR}	RF	A2780^{ZD0473R}	RF
LH1	41.1±2.90	58.2±0.79	1.4	53.2±3.06	1.3
LH2	22.7±1.60	25.5±2.45	1.1	32.9±6.5	1.5
Cisplatin	1.0±0.45	12.4±1.12	12.4	8.2±1.14	8.2
Genistein	15.02±0.05	27.80±0.09	1.85	16.24±0.07	1.1

[a]Data are the mean±standard deviation of quadruplicate determinations of at least three independent experiments. ‡Average of those obtained from four identical wells with 4,000 cells per well. RF: Ratio of IC_{50} value for A2780^{cisR} or A2780^{ZD0473R} to that for A2780 cell line.

concentrations (15 μM to 60 μM), indicates that cisplatin was much more able to prevent *BamH*I digestion than LH1 and LH2, in line with the greatest DNA conformational change induced by the compound.

Growth-inhibitory effect of single drugs. Table I lists the IC_{50} values and the resistant factor (RF), defined as the ratio of the IC_{50} value for the resistant cell line to that for the parent cell line, of LH1, LH2, cisplatin (used as a reference compound) and genistein for the ovarian cancer cell lines.

It can be seen that both LH1 and LH2 were found to be much less active than cisplatin against all three ovarian cancer cell lines, as was reported for monofunctional platinum pyriplatin (4). However, LH1 and LH2 had similar activities against both parent and resistant cell lines, unlike cisplatin which had much lower activity against the resistant cell lines. LH2 was found to be more active than LH1 even though LH1 was more damaging to pBR322 plasmid DNA and cellular DNA than LH2. The results may mean that the key determinant of cytotoxicity of LH1 and LH2 is not binding with DNA but rather is associated with their binding with other cellular targets such as RNA or proteins. If so, this would explain the observed discordance between the cytotoxicity and the level of binding with DNA. It could also explain why these compounds have similar activities against both parental and resistant cell lines. Genistein was also found to be less active than cisplatin, but like monofunctional platinums, it had lower RFs than cisplatin. Genistein can exert its cytotoxicity through the regulation of cell signalling pathways different from those of cisplatin and monofunctional platinums LH1 and LH2 (12). Relatively lower activity of genistein is not considered to be a problem as it is still considered to be sufficiently active. For example, its activity against the A2780 cell line is comparable to that of the widely used platinum drug carboplatin and much greater than that of the platinum drug against the A2780^{cisR} cell line (30).

This being an *in vitro* study, it is difficult to predict how 25 μM genistein would compare with clinically-relevant data except to state that in an 8-day steady-state pharmokinetic study of healthy post-menopausal women (n=30) randomized to receive 54 mg of genistein per day, the maximum plasma

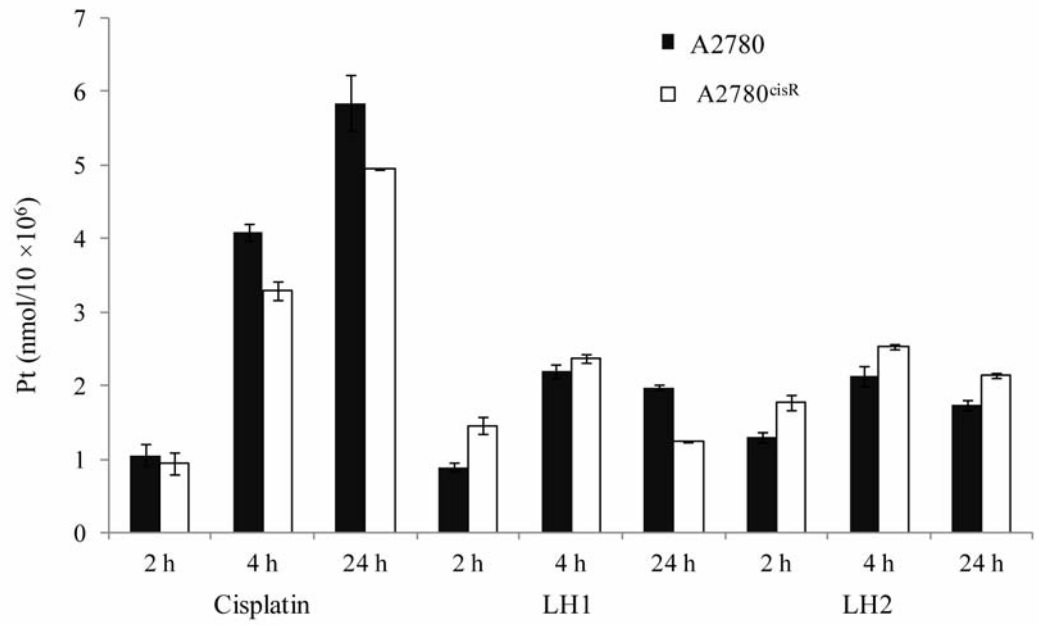

Figure 4. *Pt accumulation in A2780 and A2780cisR cell lines resulting from their incubation with 50 μM solutions of LH1, LH2 and cisplatin for 2 h, 4 h and 24 h at 37˚C.*

concentration achieved was 0.7 μM, 2 h after administration (31); it is unlikely for the maximum plasma concentration to be as high as 25 μM. Although the data provide proof-of-concept, further studies need to be carried out at clinically achievable concentrations to confirm our observations.

Cellular accumulation of Pt as a function of time. Figure 4 shows the changes in Pt accumulation in A2780 and A2780^{cisR} cell lines as a function of time on incubation with cisplatin, LH1 and LH2 at 2, 4 and 24 h (at 50 μM concentration). At 24 h, both LH1 and LH2 led to much lower levels of Pt accumulation than cisplatin in both cell lines. Whereas Pt accumulation from cisplatin increased almost linearly with time, those from LH1 and LH2 were greater at 4 h than at 24 h. Much greater accumulation of platinum from LH1 and LH2 than cisplatin at 4 h can be seen to indicate that the carrier-mediated transport for the monofunctional cationic complexes into the cell was faster than combined transport of cisplatin by all processes including passive diffusion and carrier-mediated transport. As stated earlier, positively charged monofunctional platinum complexes can be substrates for organic cationic transporters (5). Experiments to determine the effect of change in concentration of the compounds on the cellular accumulation of platinum could also have provided meaningful information.

Pt–DNA binding level. Figure 5 shows Pt–DNA binding levels in A2780 and A2780^{cisR} cells after theirincubation with solutions of compounds (at 50 μM) for 2 h, 4 h and 24 h.

In the case of cisplatin, Pt–DNA binding continued to increase with time; for LH1 and LH2, the values at 4 h were greatest (Figure 5). As noted earlier, LH1 and LH2 being positively charged would readily be attracted to the negatively charged DNA, initially undergoing associative interaction followed by covalent binding (4). The planaramine ligands in LH1 and LH2 can sterically clash with base pairs of the double helix, in effect twisting the bases out of their native conformation (5). LH1 and LH2 may also differ from cisplatin in terms of hydrogen bonding and stacking interactions. The compounds can interact with numerous cellular proteins, thereby modulating several signal transduction pathways (such as JAK-STAT signalling), thus producing cell death due to immune response (32).

Drugs in combination. CIs, median-effect dose, shape (sigmoidicity), conformity (linear correlation coefficient r) for combinations of cisplatin and genistein with LH1 and LH2 in A2780, A2780^{cisR} and A2780^{ZD0473R} cancer cell lines are given in Table II. Figure 6 provides the CI values at the median effect dose (ED_{50}).

Combinations of cisplatin with LH1 and LH2: Both bolus and sequential (0/4 h and 4/0 h) administrations of cisplatin in combination with LH1 and LH2 resulted in additivity to antagonism at low concentration against all the three human ovarian cell lines as was observed for combination of cisplatin with pyriplatin in HT29 and OVCAR-3 cell lines (4). Antagonistic to additive 0/4 h combinations of cisplatin

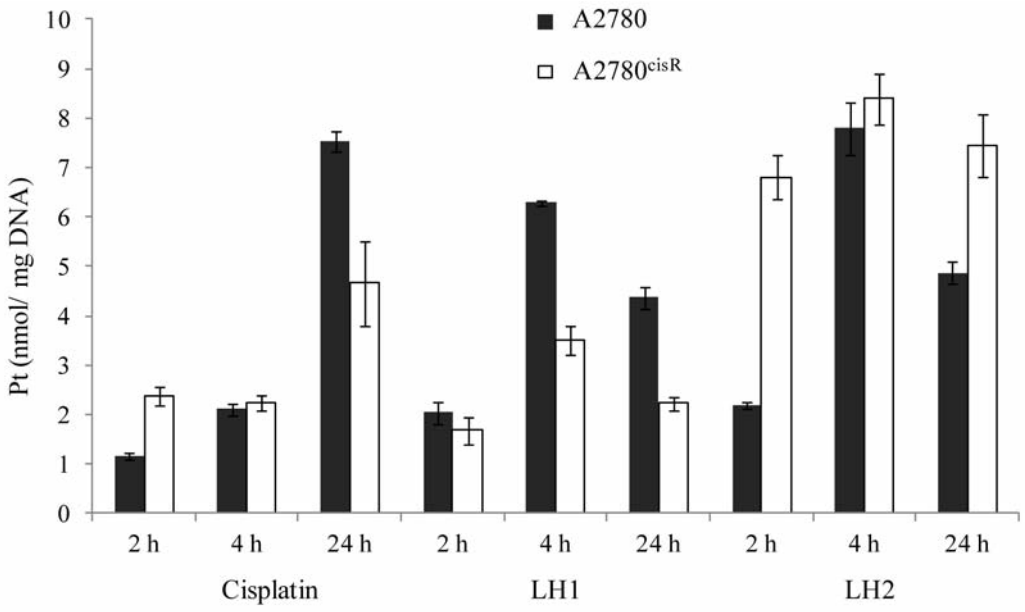

Figure 5. *Pt–DNA binding levels in A2780 and A2780^{cisR} cell lines after interaction with 50 μM solutions of LH1, LH2 and cisplatin for 2 h, 4 h and 24 h at 37˚C.*

with LH1 were found to be associated with higher Pt–DNA binding levels than slightly synergistic 0/0 h combinations against both A2780 and A2780^{cisR} cell lines, indicating a lack of association between activity and Pt–DNA binding level as applied to the combinations of monofunctional platinum LH1 with cisplatin. The decrease in Pt–DNA level with increasing incubation period indicates that DNA repair processes might be more prominent for monofunctional platinums than for cisplatin.

Combination of genistein with LH1 and LH2: Combination of genistein with LH1 and LH2 produced only weak but sequence-dependent synergism in A2780, A2780^{cisR} and A2780^{ZD0473R} ovarian cancer cell lines. A more careful analysis shows that the 0/4 h sequence of administration was most synergistic against the A2780^{cisR} cell line and the 0/0 h against the A2780^{ZD0473R} cell line. Greatest synergism observed with 0/4 h sequence of administration for combinations of LH1 and LH2 with genistein against the A2780^{cisR} cell line, indicating that pre-treatment of cells with genistein had served to sensitize them towards platinum action (33). Genistein is the predominant isoflavone found in soybeans that can inhibit growth of various cancer cells *in vitro* and *in vivo,* but without causing toxicity to normal cells (34) and it does so by regulating the genes that are involved in the control of cell proliferation, the cell cycle, apoptosis, oncogenesis, transcription regulation, angiogenesis, and cancer cell invasion and metastasis (15). It was suggested that pre-treatment of cancer cells with genistein resulted in the appearance of cleaved poly ADP ribose polymerase consistent with increased apoptosis. The hormonal actions of genistein were also suggested to be an important determinant of its anticancer action (35). It has a high affinity for binding to oestrogen receptor, particularly oestrogen receptor-β, which is involved in the suppression of oestrogen receptor-α-stimulated oestrogenic signal mechanisms. In addition to having hormonal activity, genistein exerts anti-neoplastic effects by modulating multiple signalling pathways such as protein-tyrosine kinase (PTK), AKT, NF-κB, matrix metalloproteinases, and the ratio of BAX and BCL2 (36, 37). Whereas resistance of ovarian cancer cells to cisplatin may be due to the activation of NF-κB, chemosensitization due to genistein could be due to its deactivation, resulting in the inhibition of cell proliferation and induction of apoptosis (38). Genistein has an inhibitory effect towards PTK, which drives signal transduction pathways leading to tumour growth and progression to malignancy (39). Being an antioxidant, it can also scavenge free radicals (40).

Only weak synergism resulting from combination of genistein with LH1 and LH2 but strong synergism from that with cisplatin, indicate that genistein is less effective in modulating cytotoxicity of LH1 and LH2 than that of cisplatin. This is not unexpected when we note that LH1 and LH2 differ from cisplatin in their nature of binding with DNA. Indeed the mechanisms of resistance operating in

Table II. *Dose–effect parameters applying to combinations of genistein and cisplatin with LH1 and LH2 in the A2780, A2780cisR and A2780ZD0473R cell lines.*

Drug	Sequence (h)	A2780					$A2780^{cisR}$					$A2780^{ZD0473R}$				
		Molar ratio	CIs at ED_{50}	D_m	m	r	Molar ratio	CIs at ED_{50}	D_m	m	r	Molar ratio	CIs at ED_{50}	D_m	m	r
Cisplatin				0.46	0.33	1.00			11.30	0.66	0.98			8.52	0.77	0.99
LH1				28.27	0.51	0.95			38.98	0.77	0.90			42.63	1.09	0.96
Cisplatin	0/0		1.65	0.42	0.42	0.70		0.90	4.51	0.74	0.98		0.84	3.78	0.88	0.98
+	0/4	1:50.38	1.50	0.38	0.38	0.68	1:4.34	1.00	5.02	0.93	0.99	1:4.54	1.06	4.78	1.05	0.98
LH1	4/0		1.42	0.36	0.36	0.67		1.17	5.87	0.85	0.99		1.06	4.77	0.92	0.99
LH2				0.45	0.36	1.00			11.30	0.33	0.98			28.6	1.07	0.97
Cisplatin	0/0		1.32	0.28	0.62	1.00		0.90	4.51	0.74	0.98		0.91	4.30	0.94	0.99
+	0/4	1:26.57	0.98	0.21	0.63	1.00	1:2.39	1.00	5.01	0.93	0.98	1:2.72	0.94	4.40	0.96	0.99
LH2	4/0		0.81	0.17	0.56	1.00		1.17	5.87	0.85	0.99		0.93	4.37	0.86	0.99
Genistein				16.67	0.71	1.00			16.84	0.91	0.99			40.0	1.10	0.94
LH1				48.69	0.94	1.00			66.89	1.09	0.98			55.2	1.16	0.98
Genistein	0/0		0.91	7.11	0.89	0.99		0.86	9.19	0.91	0.99		0.78	8.21	1.10	0.99
+	0/4	1:1.34	0.92	7.12	0.77	0.97	1:2.31	0.48	5.15	0.52	0.99	1:3.9	0.87	9.11	1.11	0.99
LH1	4/0		0.97	7.57	0.81	0.95		0.61	6.48	0.48	0.98		0.87	9.14	1.15	0.98
LH2				30.53	0.62	0.99			43.91	1.11	0.99			54.49	1.42	0.99
Genistein	0/0		0.85	7.39	0.74	1.00		0.76	8.56	0.70	0.99		0.79	11.8	1.00	0.99
+	0/4	1:1.12	1.12	9.70	0.79	1.00	1:1.27	1.44	16.29	0.84	0.99	1:2.34	1.10	16.37	1.89	0.99
LH2	4/0		1.32	11.46	0.89	0.99		2.25	15.48	1.86	0.96		1.13	16.7	1.86	0.98

ED_{50}: Effective dose for 50% cell kill; Dm: median effect dose, m: exponent defining shape of the dose effect curve; r: reliability coefficient.

$A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines are characteristic of cisplatin and its analogues, so that there is a large reduction in activity of cisplatin and its analogues in going from A2780 to $A2780^{cisR}$ and $A2780^{ZD0473R}$ cells, whereas LH1 and LH2 have comparable activity in the parent as well as the resistant cell lines.

Cellular accumulation of platinum from combination treatments. Table III provides platinum accumulation in A2780 and $A2780^{cisR}$ cells after incubation with combinations of cisplatin and genistein with LH1 and LH2 for 24 h at 37˚C, administered using 0/0 h, 0/4 h and 4/0 h sequences. It can be seen that generally higher intracellular platinum accumulation resulted from combinations of LH1 and LH2 with genistein than from platinum compounds alone (and more so in the resistant $A2780^{cisR}$ cell line), irrespective of whether the combined drug action was synergistic or not. It was the 4/0 h combination of genistein with LH1 that resulted in highest platinum accumulationin $A2780^{cisR}$ cell line.

The higher accumulation of platinum from combinations of LH1 and LH2 with genistein possibly indicates that the presence of genistein enhanced the rate of carrier-mediated transport of LH1 and LH2 into the cells. Similarly it was reported that the presence of genistein increased the accumulation of cisplatin into cells (15, 33, 35). The highest platinum accumulation from 4/0 h combination of LH1 and genistein in $A2780^{cisR}$ cells indicates that the pre-treatment of cells with genistein for 4 h before administration of LH1 was most effective in increasing platinum accumulation and enhancing cell kill. However, the increase in platinum accumulation was not associated with corresponding increase in Pt–DNA binding although the level achieved in the resistant cell line was greater than that in the parental cell line. Discordance between Pt accumulation and Pt–DNA binding, more importantly that between Pt–DNA binding level and the extent of cell kill can be seen to indicate that DNA alone may not be the critical target for LH1 responsible for causing cell death. It has been reported that platinum drugs can also bring about cell death that is immunological independent of binding with DNA (32). In agreement with this idea, synergistic from 4/0 h combination of LH1 with genistein in the $A2780^{cisR}$ cell line was associated with high intracellular platinum accumulation. However, antagonistic 4/0 h combination of LH2 with genistein in $A2780^{cisR}$ cell line was also found to be associated with moderately high intracellular platinum accumulation. The results can be seen to indicate both the nature of the combined drug action and the level of intracellular Pt accumulation are also a function of the nature of planaramine ligand present. Whereas the carrier ligand in LH1 is 3-hydroxypyridine, that in LH2 is imidazole.

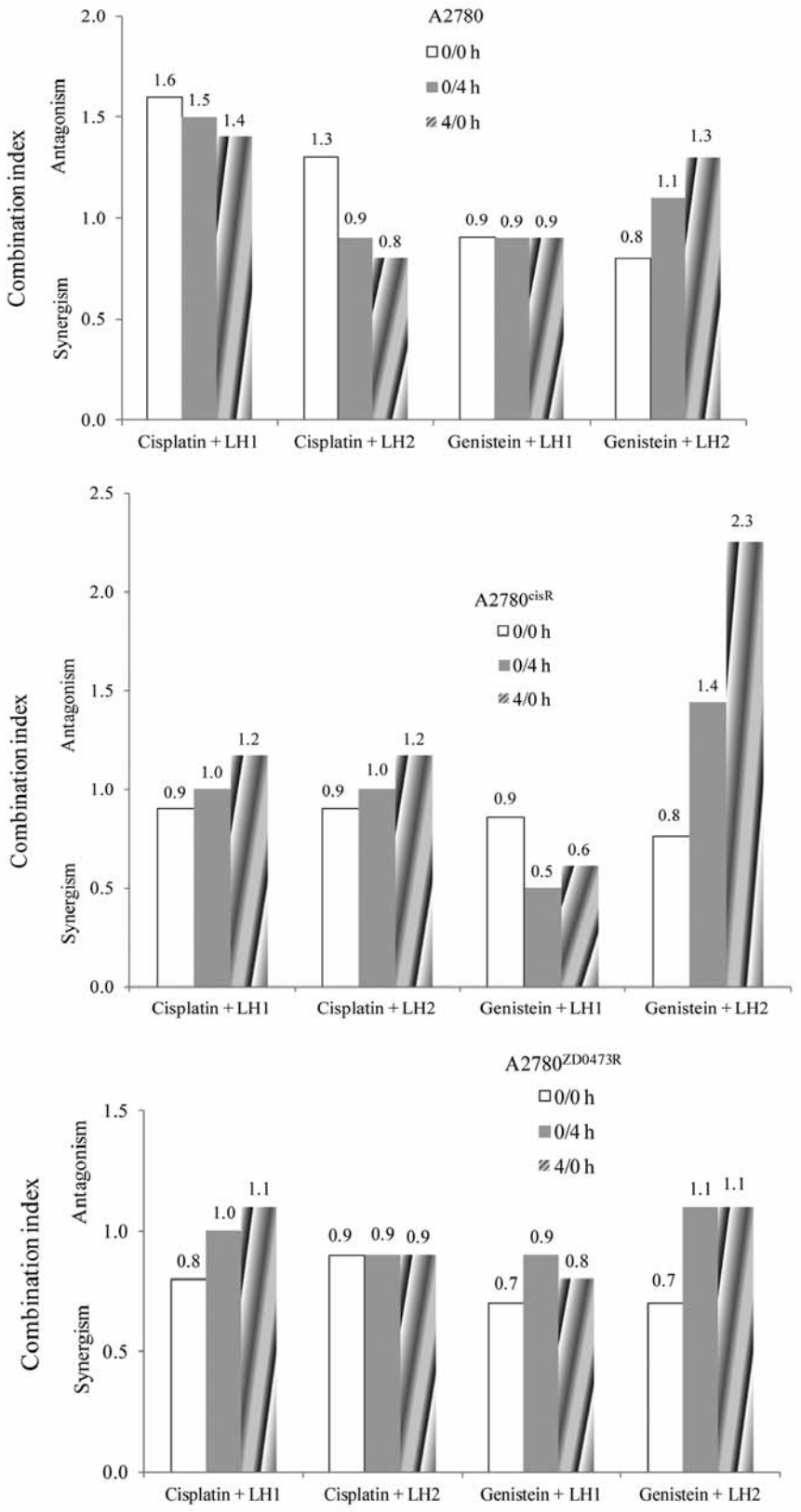

Figure 6. *Combination indices applying to the 0/0 h, 0/4 h and 4/0 h combinations of cisplatin and genistein with LH1 and LH2 at the median effect dose in A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines (based on the pooled data from three to five individual experiments).*

Table III. *Platinum accumulations in A2780 and A2780cisR cells after their interaction with combinations of cisplatin and genistein with LH1 and LH2 (at 25 μM concentration) for 24 h at 37˚C, administered using 0/0 h, 0/4 h and 4/0 h sequences*

Drug	Sequence	Combined drug effect*		Platinum accumulation (nmol Pt/5×10^6 cells)	
		A2780	A2780^{cisR}	A2780	A2780^{cisR}
Cisplatin				5.84±0.38	4.95±0.02
LH1				0.28±0.03	1.14±0.01
LH2				0.74±0.06	0.40±0.03
Cisplatin + LH1	0/0 h	Antagonistic	Nearly additive	3.75±0.36	4.55±0.29
	0/4 h	Antagonistic	Nearly additive	3.70±0.49	19.79±0.67
	4/0 h	Moderately antagonistic	Slightly antagonistic	4.81±0.45	4.82±0.22
Cisplatin + LH2	0/0 h	Moderately antagonistic	Nearly additive	8.47±0.56	8.82±0.89
	0/4 h	Nearly additive	Nearly additive	6.84±0.53	8.19±0.81
	4/0 h	Moderately synergistic	Slightly antagonistic	6.43±0.34	7.98±0.47
Genistein + LH1	0/0 h	Nearly additive	Slightly synergistic	1.25±0.10	3.61±0.24
	0/4 h	Nearly additive	Synergistic	1.45±0.04	12.61±1.17
	4/0 h	Nearly additive	Synergistic	3.34±0.37	42.79±3.45
Genistein + LH2	0/0 h	Slightly synergistic	Moderately synergistic	6.17±0.36	3.60±0.36
	0/4 h	Slightly antagonistic	Moderately antagonistic	1.08±0.10	3.69±0.35
	4/0 h	Moderately antagonistic	Antagonistic	3.31±0.24	8.27±0.82

*Based on combination indices at effective dose for 50% cell kill.

Table IV. *Levels of platinum–DNA binding in A2780 and A2780^{cisR} cells after their incubation with combinations of cisplatin and genistein with LH1 and LH2 (at 25 μM concentrations) for 24 h at 37˚C, using 0/0 h, 0/4 h and 4/0 h sequences of administration*

Drug	Sequence	Combined drug effect*		Pt–DNA binding level (nmol Pt per mg of DNA	
		A2780	A2780^{cisR}	A2780	A2780^{cisR}
Cisplatin				7.53±0.19	4.66±0.86
LH1				4.37±0.22	2.22±0.14
LH2				4.87±0.22	7.44±0.64
Cisplatin + LH1	0/0 h	Antagonistic	Nearly additive	2.87±0.22	6.47±0.34
	0/4 h	Antagonistic	Nearly additive	8.07±0.51	7.74±0.65
	4/0 h	Moderately antagonistic	Slightly antagonistic	4.86±0.48	6.66±0.60
Cisplatin + LH2	0/0 h	Moderately antagonistic	Nearly additive	4.48±0.46	3.50±0.20
	0/4 h	Nearly additive	Nearly additive	3.56±0.34	5.45±0.38
	4/0 h	Moderately synergistic	Slightly antagonistic	4.15±0.34	4.38±0.41
Genistein + LH1	0/0 h	Nearly additive	Slightly synergistic	1.18±0.05	1.11±0.07
	0/4 h	Nearly additive	Synergistic	1.10±0.09	2.05±0.16
	4/0 h	Nearly additive	Synergistic	0.93±0.13	1.78±0.15
Genistein + LH2	0/0 h	Slightly synergistic	Moderately synergistic	1.20±0.04	1.05±0.03
	0/4 h	Slightly antagonistic	Moderately antagonistic	1.31±0.13	1.57±0.18
	4/0 h	Moderately antagonistic	Antagonistic	1.15±0.16	1.15±0.05

*Based on combination indices at effective dose for 50% cell kill.

Pt–DNA binding from combination treatments. Table IV gave the Pt–DNA binding levels in A2780 and A2780^{cisR} cells after interaction with combinations of cisplatin and genistein with LH1 and LH2 for 24 h at 37˚C, using 0/0 h, 0/4 h and 4/0 h sequences of administration. It can be seen that when cisplatin was administered in combination with LH1, a higher level of Pt–DNA binding resulted in the resistant A2780^{cisR} cell line from the combination than from cisplatin alone, irrespective of the sequence of administration. For the combination of cisplatin with LH2, the level of Pt–DNA binding using the 0/4 h sequence of administration was significantly greater in the resistant A2780^{cisR} cell line than in the parental A2780 cell line.

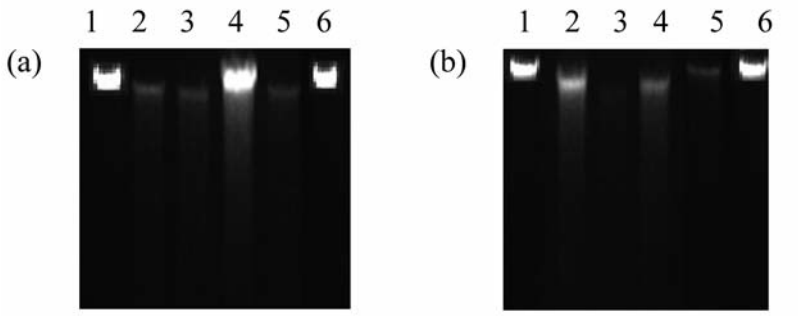

Figure 7. *Agarose gel electrophoresis of nuclear DNA isolated from A2780 (a) cells and A2780^{cisR} (b) cells after their incubation with selected combinations of cisplatin with LH1 and LH2. Lanes 1 and 6: Control A2780 or A2780^{cisR}, lane 2: cisplatin + LH1 0/0 h, lane 3: cisplatin + LH1 0/4 h, lane 4: cisplatin + LH1 4/0 h, lane 5: cisplatin + LH2 0/4 h.*

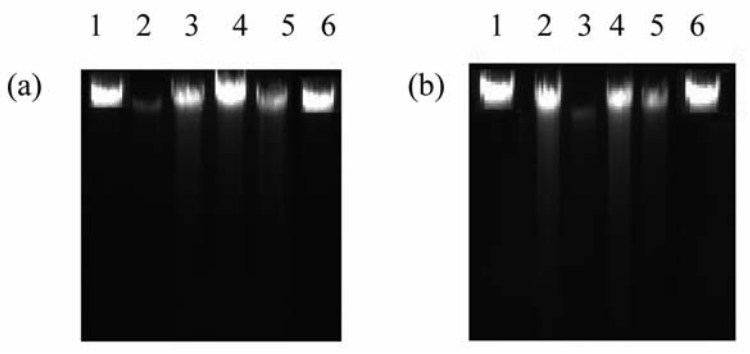

Figure 8. *Agarose gel electrophoresis of nuclear DNA isolated from A2780 (a) cells and A2780^{cisR} (b) cells after their incubation with selected combinations of genistein with LH1 and LH2. Lanes 1 and 6: Control A2780 or A2780^{cisR}, lane 2: genistein + LH1 0/4 h, lane 3: genistein + LH1 4/0 h, lane 4: genistein + LH2 0/4 h, lane 5: genistein + LH2 4/0 h.*

When LH1 was administered in combination with genistein, the 0/4 h sequence of administration resulted in the highest level of Pt–DNA binding in the resistant cell line followed by that of the 4/0 h sequence of administration. For the combination of genistein with LH2, it was also the 0/4 h sequence of administration that resulted in the highest the level of Pt–DNA binding. Although no clear trend in Pt–DNA binding levels from the combination of cisplatin with LH1 and LH2 or that of genistein with LH1 and LH2 could be seen for A2780 and A2780cisR cells, synergistic 0/4 h and 4/0 h combinations of LH1 with genistein were found to be associated with higher Pt–DNA binding levels in the resistant A2780^{cisR} cell line.

DNA fragmentation. Figures 7 and 8 show the bands in agarose gel electrophoresis of DNA isolated from A2780 and A2780cisR cells before and after their incubation with selected combinations of cisplatin and genistein with LH1 and LH2.

It can be seen that in both A2780 and A2780cisR cell lines, the 0/4 h combination of cisplatin with LH1 and LH2 were more damaging to nuclear DNA; in the A2780 cell line 0/0 h, the combination of cisplatin with LH1 was also highly damaging to DNA. For the combination of genistein with LH1 and LH2, in the A2780 cell line, the 0/0 h combination of genistein with LH1 was most damaging and in the A2780^{cisR} cell line it was the 4/0 h combination of genistein with LH1 that was most damaging followed by the 4/0 h combination of genistein with LH2. The results can be seen to indicate that LH1 was more damaging to cellular DNA than LH2 even though it was less active than LH2. As noted earlier, the lack of association between activity and Pt–DNA binding and that between activity and DNA damage highlight that the cytotoxic activity of LH1 and LH2 (alone and in combination with genistein) is not limited to their DNA binding. As noted earlier, the compounds may also induce cell death *via* DNA-independent pathways.

Conclusion

Although the compounds LH1 and LH2 were found to be less active than cisplatin, they produced synergistic outcomes in combination with genistein. The lack of association

between activity and the level of Pt–DNA binding and that between activity and DNA damage can be seen to indicate that the compounds are also able to cause cell death *via* DNA-independent pathways.

Acknowledgements

The Authors are thankful to Dr. Ian Luck of the School of Chemistry, The University of Sydney, Australia for recording ^{1}H NMR spectra. Laila Arzuman is grateful to The University of Sydney for the award of a UPA Scholarship. This project is supported by Biomedical Science Research Initiative Grant and Biomedical Science Cancer Research Donation Fund.

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Received July 8, 2015
Revised September 4, 2015
Accepted September 10, 2015