Synergism from Combinations of *tris*(benzimidazole) monochloroplatinum(II) Chloride with Capsaicin, Quercetin, Curcumin and Cisplatin in Human Ovarian Cancer Cell Lines

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Abstract. *In the present study, synergism in activity from the sequenced combinations of monofunctional platinum tris(benzimidazole)monochloroplatinum(II) chloride (coded as LH4) with capsaicin, quercetin, curcumin and cisplatin was investigated as a function of sequence of administration in a number of human ovarian tumor models. Cellular accumulations of platinum and the levels of platinum–DNA binding were also determined for the 0/0 h and 4/0 sequences of administration. LH4 was found to be more active against the resistant A2780^{cisR} and A2780^{ZD0473R} cell lines than the parent A2780 cell line. As applied to combinations of LH4 with phytochemicals capsaicin, quercetin and curcumin, bolus administration was found to be most synergistic in both the parent A2780 and the resistant A2780^{cisR} cell lines. For the combinations of LH4 with cisplatin, additiveness was observed in both the resistant cell lines but mild synergism was observed in the parent cell line. Greater activity of designed monofunctional platinum LH4 against resistant tumor models and synergism from combinations with phytochemicals indicate that the compound has the potential for development as a novel platinum-based anticancer drug.*

Ovarian cancer is the leading cause of death from gynecological cancers in women in the Western world, for which the overall success rate remains low) (1). Combination of cisplatin (or carboplatin) and paclitaxel are the standard choice of chemotherapy in ovarian cancer. However, platinum resistance (acquired or intrinsic) continues to remain as a major problem (2). Because of the lack of early symptoms, most patients would be at an advanced stage at the time of initial diagnosis and they die of recurrence of the tumour that is resistant to conventional treatment (3-5). Thus, there is a need for new platinum-based drugs with novel mechanisms of action and use of different treatment regimens consisting of drug combinations to improve therapeutic outcome. Monofunctional cationic platinum complexes that contain only one site for binding with DNA have been developed, characterized with a greater efficacy than cisplatin and oxaliplatin in killing cancer cells and inhibiting transcription (6). In the present study we report the synthesis and activity of the monofunctional platinum(II) complex *tris*(benzimidazole)chloroplatinum(II) [coded as LH4] (Figure 1) against ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cancer cell lines. Damage to cellular DNA was also investigated.

The effect of combination of LH4 with the phytochemicals capsaicin (Caps), quercetin (Quer) and curcumin (Cur) (Figure 1) and that with cisplatin (CS) was also investigated with the idea that the compounds together might be better able to overcome platinum resistance. Caps is a major pungent smelling compound found in red pepper and hot chilli that belong to the plant genus *Capsicum* (Solanaceae) (7, 8). It has long been reported to control obesity and possess anti-carcinogenic and chemo-preventive activities (9, 10). It has been recently reported that Caps can inhibit the growth of tumor cells and induce apoptosis in various cancer cells including the human leukemia HL-60 cell line (11), gastric adenocarcinoma cell line (AGS cells) (12) and human breast cancer MCF-7 cell line (12). It is a chemopreventive that is able to inhibit multi-stage carcinogenesis (13) and angiogenesis *in vitro* and *in vivo* (14) and does so by blocking the translocation of nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1) and signal transducer and activator of

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Key Words: Benzimidazole, monofunctional platinum, drug resistance, drug combination, ovarian cancer.

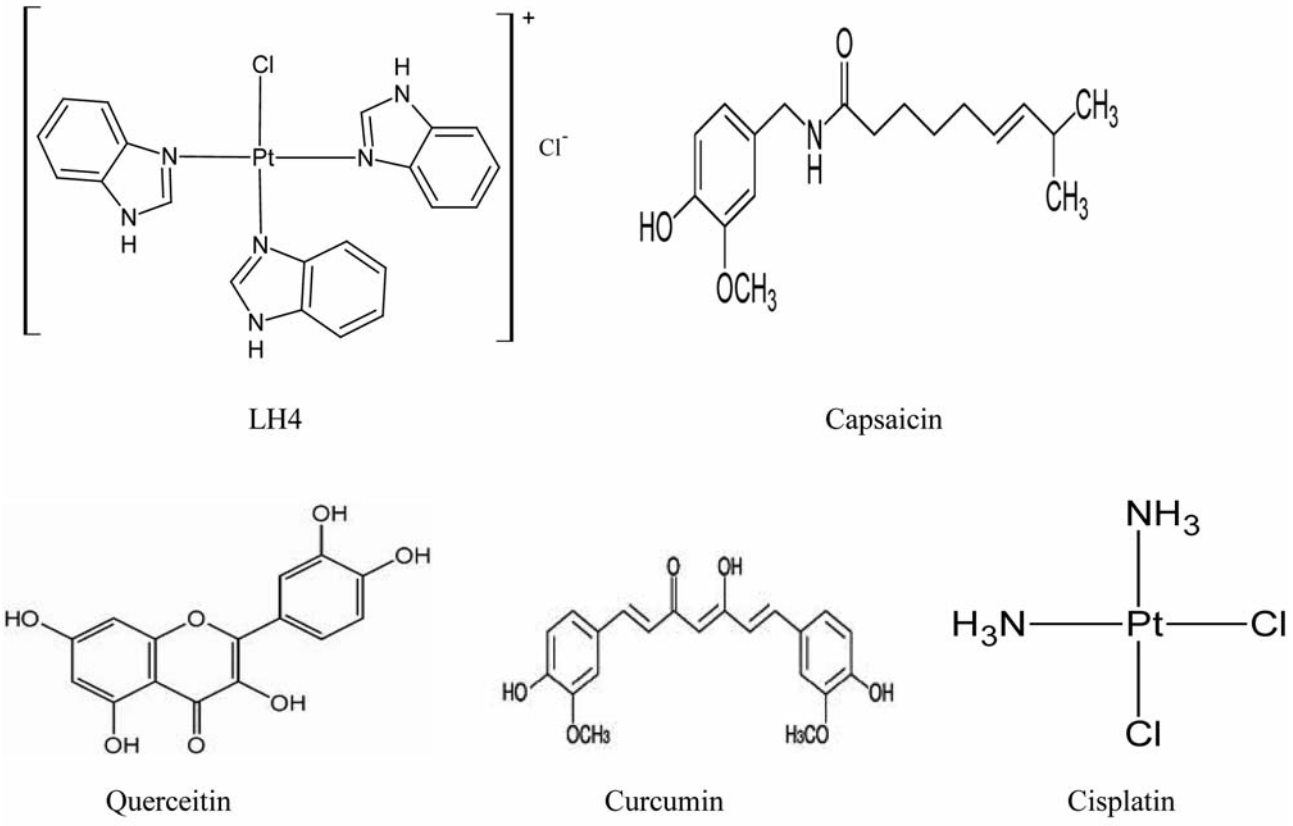

Figure 1. *Structures of LH4, capsaicin, quercetin, curcumin and cisplatin.*

transcription (STAT3) signaling pathway that are required for carcinogenesis (7, 15).

Quer is a flavanoid that serves as an antioxidant (Figure 1). It is ubiquitously present in such fruits as cranberries, elderberries and red apples, vegetables such as onions, seeds, nuts and tea (16, 17). Quer displays a variety of biological activities including chemoprevention and inhibition of tumour growth (18). Quer also increases intracellular reactive oxygen species (ROS) levels due to production of quercetin radicals (Quercetin-O•) during its reaction with peroxyl radicals (19), thus causing free radical-induced apoptosis through ROS/AMPKα1/ASK1/p38 and the AMPKα1/COX2 signaling pathways (20). Quercetin radicals are also found to lower the intracellular glutathione (GSH) pool in a concentration-dependent manner (20) and trigger apoptosis through mitochondrial depolarization (21). Quer is known to interact with type II estrogen binding site. Quer has been reported to act synergistically in combination with cisplatin in killing ovarian cancer cells such as OVCA433, CAOV3 and SKOV3 (5).

Cur is a polyphenol from rhizome of the plant *Curcuma longa* (Figure 1). It is a key component of turmeric that has antioxidant, anti-toxic, anti-inflammatory, cancer chemopreventive and potentially chemotherapeutic properties (5, 22). Recent studies have demonstrated that these diverse activities of Cur are mediated through multiple signaling pathways (23) and that the molecular targets of Cur include transcription factors, growth factors, cytokines, enzymes and other gene products (24). Cur has been shown to inhibit the FA/BRCA pathway and sensitize CAOV3 and SKOV3 ovarian cancer cells to cisplatin-induced apoptosis (25, 26). Because of differences in their mechanism of action, it is logical to think that combination of monofunctional platinum LH4 with phytochemicals Caps, Quer and Cur may exhibit sequence-dependent synergism. To the best of our knowledge, this is the first report on combination between monofunctional platinum with Caps, Quer, Cur in ovarian cancer cell lines.

Materials and Methods

Synthesis and characterization

Materials. Potassium tetrachloroplatinate (II) ($K_2[PtCl_4]$) was from Sigma Chemical Company St. Louis, MO, USA); N,N-dimethylformamide [DMF] [C_3H_7NO] and benzimidazole [$C_7H_6N_2$] from MP Biomedicals, LLC, Solon, France; HCl from Ajax chemicals, Auburn NSW, Australia and ethanol from Merck Pty Ltd., Kilsyth, Australia.

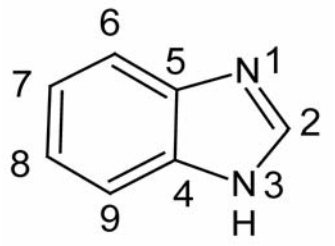

Figure 2. *Numbering scheme adopted for benzimidazole ligand.*

Method. Half mmol of K_2PtCl_4 (0.210 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. Five millimoles of benzimidazole (0.6 g) dissolved in a mixture of 2 ml of DMF and 2 ml of mQ water were added dropwise over a period of 2 h to the solution of potassium tetrachloroplatinate. The reaction mixture, protected from light, was stirred at room temperature for 14 days after which 25 ml of 0.25 M hydrochloric acid was added to the mixture. Stirring was continued for 1 week at room temperature in dark. The mixture was then left standing for 24 h at room temperature. It was then centrifuged at 5,500 rpm for 10 min to collect precipitate of LH4. The crude product of LH4 was washed with ice cold ethanol and purified by precipitation from 0.05 M HCl. The yield of the product was 58% (180 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 (supplier, address). Pt was determined by graphite furnace atomic absorption spectroscopy (AAS). As LH4 could not be obtained in crystalline form, infrared spectroscopy (IR), mass spectrum (MS) and proton nuclear magnetic resonance (1H NMR) spectra were used to aid in its structural characterization. The IR spectrum was obtained using a Varian Fourier transform infrared (FT-IR) spectrometer (Bruker IFS66 spectrometer, address). To obtain the mass spectrum, solution of LH4 made in 90% methanol and 10% DMF was sprayed into a Finnigan LCQ mass spectrometer. To obtain ^{1}H NMR spectrum, the compound was dissolved in deuterated dimethyl sulfoxide (DMSO) 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 benzimidazole ligand which is given below:

LH4: As a pale yellow powder (180 mg, 58 %); ^{1}H NMR (400 MHz, [D_6] DMSO: δ (ppm)=9.1 (d, due to C_5H), 8.24 (d, due to C_4H), 8.21 (s, due to NH), 7.65 (q, due to C_7H), 7.32 (s, due to C_2H), 7.2 (q, due to C6H); 3.69 (s, due to water), 2.49 (s, due to DMSO); IR (KBr): 2360, 2342, 1750, 1510, 1233, 1150, 1000, 747, 665 cm^{-1}; MS (ESI) *m/z* (%): 666 (3.0)=[Pt $(C_7H_6N_2)_3Cl_2$ + Cl + 8H], 584.09 (5.85)=[Pt $(C_7H_6N_2)_3Cl_2$ + Cl], 333.58 (3.0)=[Pt $(C_7H_6N_2)_3Cl_2$ + $(C_7H_6N_2)_2$ − Cl − NH_2]; Calculated combustion element analysis (Anal. calcd) for $C_{21}H_{18}Cl_2N_6Pt$: C 40.5, H 2.9, N 13.6, Pt 31.5, found: C 40.1±0.4, H 2.5±0.4, N 13.2±0.4, Pt 31.6±1.0.

Molar conductivity. Molar conductivity values for solutions of LH4 and 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 LH 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

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 (27, 28). Briefly, 4,000 to 5,500 cells (maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated fetal bovin serum (FBS), 20 mM HEPES, 0.112% sodium bicarbonate and 2 mM glutamine without 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% 10% FCS/RPMI-1640 medium (0.16 to 20 μM for CS and LH4, 0.16 to 200 μM for Caps, Quer and Cur), 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 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 LH4 was added first followed by the addition of phytochemical (Caps/Quer/Cur) or CS 4 h later and 4/0 h meant the converse. The concentration ranges were: LH4: 0.74-11.76 μM, 1.02-16.38 μM and 0.66-10.47 μM; Caps: 3.60-57.66 μM, 3.12-49.90 μM and 4.14-66.18 μM; Quer: 1.90-30.43 μM, 3.55-56.80 μM and 2.42-38.63 μM; Cur: 1.1-17.63 μM, 1.34-21.41 μM and 1.35-21.52 μM; CS: 0.10-1.59 μM, 1.24-19.84 μM and 0.90-14.40 μM for the 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 (27, 28).

Median effect analysis (29), 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 combination index (CI) as a quantitative measure of combined action using the Calcusyn software (V2) (Biosoft, city, UK).

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

where D_1 and D_2 in the numerator stand for the concentrations of compounds 1 and 2 in combination needed to achieve x% inhibition whereas D_{1x} and D_{2x} in the denominator represent the same when they are present alone. In the following equation, D_x denotes dose of drug, D_m is the median-effect dose which is equivalent to 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.

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

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.

Platinum accumulation and Pt–DNA binding. Cellular accumulation of platinum and platinum–DNA binding levels were measured in order to determine whether the values were affected by the sequence of addition or there was any correlation between the synergistic action and the level of Pt–DNA binding. As applied to the combinations of LH4 with Caps, Quer, Cur and CS, these were done for most synergistic (0/0 h) and most antagonistic (4/0 h) combinations. It is believed that the results may aid in the understanding of combined drug action. The method used for the determination of total intracellular platinum and platinum–DNA level was a modification of that described by Di Blasi *et al*. (30). LH4, Caps, Quer, Cur and CS were added to culture plates containing exponentially growing A2780 and A2780^{cisR} cells in 5 ml 10% FCS/RPMI 1640 culture medium (cell density=1×10^6 cells ml^{-1}). The cells containing the drugs were incubated for 24 h at the end of which cell monolayers were trypsinized and cell suspension (5 ml) was transferred to centrifuge tube and spun at 3,500 rpm for 2 min at 4˚C. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at −20˚C until assayed.

Cellular accumulation. Cell pellets from drug combinations were suspended in 0.5 ml 1% triton-X, held on ice while being sonicated. Total intracellular platinum contents were determined by a graphite furnace AAS using a Varian SpectrAA-240 plus with a GTA 120 atomic absorption spectrophotometer.

Platinum–DNA binding. DNA was isolated from cell pellet using the H440050 JETQUICK Blood DNA Spin Kit/50 (Austral Scientific Pty Ltd., Taren Point NSW, Australia) according to the modified protocol of Bowtell (31). DNA content was determined by UV spectrophotometry (260 nm) (Varian Cary 1E UV-Visible with Varian Cary Temperature Controller) and platinum level was determined by graphite furnace AAS. A_{260}/A_{280} ratios, found to be between 1.75 and 1.8 for all DNA samples, ensured their high purity, and the DNA concentration was calculated according to the equation: Concentration=Absorbance at 260 nm × 50 ng/μl.

DNA fragmentation. DNA isolated from A2780 and A2780^{cisR} cells after their interaction in combination of LH4 with Quer, Cur and CS (as described earlier) using 4/0 h sequences of administration and 0/0 h sequence of administration in the case of combination of LH4 with Caps for 24 h, were subjected to agarose gel (2%) electrophoresis containing ethidium bromide (32) . The amount of the DNA was kept constant (at 0.5 μg) for each drug. DNA bands were viewed under UV light and photographed as described previously.

Results and Discussion

Chemistry. LH4 (*tris*(benzimidazole)chloroplatinum(II)) was synthesized according to the modified Kauffman method (33). Cisplatin, used as a reference compound, was synthesized according to Dhara's method (34). Synthesis and characterization were fully described in the experimental section. Structures of LH4, cisplatin (CS), capsaicin (Caps), querceitin (Quer) and curcumin (Cur) are shown in Figure 1.

Molar conductivity. The molar conductivity values of the designed platinum compound, LH4, was determined to obtain information on the extent of their dissociation in solution in water that in turn are expected to provide information on their likely modes of transport across the cell membrane. Whereas non-polar and non-ionic substances can cross the cell membrane by both passive diffusion and carrier-mediated transport (such as facilitated diffusion and active transport) (35), polar molecules and charged species usually cross the cell membrane by carrier-mediated transports (35, 36). Substances can also cross the cell membrane by endocytosis (cells engulfing a plug of solid) and pinocytosis (cells engulfing a plug of liquid). As cisplatin is administered by intravenous route, the molecule is expected to remain largely undissociated in the extracellular fluids that contain much higher concentrations of chlorides (35). Thus, cisplatin may cross the cell membrane by both passive diffusion and carrier-mediated transport (35). It was suggested in a previous study that cisplatin can also cross the cell membrane by pinocytosis (37). Unlike cisplatin, designed monofunctional platinum compound LH4, being ionic in nature, is expected to cross the cell membrane by carrier-mediated transport only.

The limiting molar conductivity values (Λ_0) for CS and LH4 were found to be 136 and 368 Ω^{-1} cm^2 mol^{-1} respectively. Higher value for LH4 as compared to CS can be seen in agreement with the ionic nature of the compound (38).

Growth inhibitory effect of single drugs. Table I lists the IC_{50} values and the resistant factors (RF) of LH4, CS (used as a reference compound), Caps, Quer and Cur as applied 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 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.

Although LH4 is less active than CS against the parent cell line, it is found to be more active than CS against both the resistant cell lines indicating that LH4 must differ from CS in the mechanisms of anti-tumour action and possess a greater ability than CS to overcome drug resistance. As LH4 contains three benzimidazole carrier ligands as opposed to two ammonia ligands in CS, it can undergo distinctly different non-covalent interactions in terms of hydrogen bonding and more importantly stacking interaction in between nucleobases in the DNA. These may have a significant bearing on its ability to induce cell kill, possibly due to differences in protein recognition. Recently it has been reported that monofunctional cationic compounds containing bulky planaramine ligand can kill cancer cells with a greater efficacy than cisplatin (CS) and oxaliplatin (6, 38).

Among the phytochemicals, Cur is found to be most active and Caps the least against all the three cell lines. The lower RF values for the phytochemicals (2 or less) than for CS (about 12) indicate that the compounds were better able to induce cell death than CS in the resistant cell lines.

Drugs in combination. The combined drug action in terms of combination indices (CIs), median-effect dose, shape (sigmoidicity) and conformity (linear correlation coefficient r) applying to the combinations of LH4 with Caps, Quer and Cur administered to A2780, A2780^{cisR} and A2780^{ZD0473R} cancer cell lines using 0/0 h, 0/4 h and 4/0 h sequences of administration are given in Table II (0/0 h meant that both the drugs were administered at the same time, 0/4 h meant that LH4 was administered first followed by phytochemical or CS 4 h later and 4/0 h meant the converse). Figure 3 provides a plot of CI values at the median effect dose (ED_{50}) for the combinations using the three sequences of administration.

The results show that, as applied to the combinations of LH4 with Caps administered to A2780, A2780^{cisR} and A2780^{ZDO473R} cell lines, although all the three sequences of administration resulted in synergistic outcomes, it was the bolus administration that produced greatest synergism whereas the 0/4 h sequence of administration produced lowest synergism. In actual fact, 0/4 h combinations of LH4 with Cur were close to being additive in A2780^{cisR} and A2780^{ZDO473R} cell lines. Greater variation of combination index with the sequence of administration as applied to the combination of LH4 with Caps in A2780^{cisR} and A2780^{ZDO473R} cell lines than in the parent A2780 cell line indicates that the appropriate choice of sequence of administration is more critical in the resistant cell lines than in the parent cell line. This means that to better-overcome mechanisms of resistance operating in A2780^{cisR} and A2780^{ZDO473R} cell lines, we need to administer LH4 and Caps together rather than with a time gap. As applied to the combinations of LH4 with Quer also, the bolus administration was found to be most synergistic in A2780^{cisR} and A2780^{ZD0473R} cell lines (whereas it was the 0/4 h sequence of administration that was most synergistic in A2780 cell line) again highlighting that the two compounds need to administered at the same time to better-overcome the mechanisms of resistance operating in the cell lines. As applied to the combinations of LH4 with of Cur, all three sequences of administration were found to be almost equally synergistic in the A2780 cell line, whereas it was the 4/0 h sequence of administration that was most synergistic in A2780^{cisR} cell line and the 0/0 h sequence of administration that was most synergistic in A2780^{ZD0473R} cell line. The results indicate that, as applied to the combinations of LH4 with Cur, pre-treatment (or co-treatment) along with the phytochemical is needed to sensitize the cancer cells to platinum action. As applied to the combinations of LH4 with CS, the 4/0 h sequence of administration was most synergistic in A2780 and A2780^{ZD0473R} cell lines, whereas in A2780^{cisR} cell line all three sequences of administration were found to be almost equally synergistic. Before we more fully discuss the mechanisms of combined drug action, it is appropriate to consider the results on cellular accumulation of platinum and the level of platinum–DNA binding.

Table I. *IC_{50}[a] values (μM) and resistance factors (RF) for LH4, Caps, Quer, Cur and CS (used as a reference compound) as applied to the human ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}[b].*

Drugs	A2780	A2780^{cisR}	RF[c]	A2780^{ZD0473R}	RF[d]
LH4	7.35±0.35	10.24±2.51	2.89	6.54±1.18	0.89
CS	1.0±0.45	12.4±1.12	12.4	8.2±1.14	8.2
Caps	36.04±1.0	31.19±1.2	0.86	41.36±0.09	1.14
Quer	19.02±0.05	35.50±0.07	1.86	24.14±0.06	1.27
Cur	11.02±0.09	13.38±0.08	1.21	13.45±0.09	1.22

[a] The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments and are given±standard deviation (SD). [b] The results are averages of those obtained from four identical wells with 4,000 cells per well. [c] Ratio of IC_{50} value in A2780cisR to A2780 cell line. [d] Ratio of IC_{50} value in A2780ZD0473R to A2780 cell line.

Cellular platinum accumulation and platinum–DNA binding level. Figure 4 gives the total intracellular platinum in A2780 and A2780^{cisR} cells after their interaction with combinations of LH4 with Cur, Caps, Quer and CS (at 25 μM concentrations) for 24 h at 37˚C, administered using 0/0 h and 4/0 h sequences where 0/0 h meant that both the compounds were administered at the same time, 0/4 h meant that LH4 was administered first followed by the phytochemical or CS 4 h later and 4/0 h meant the converse. The results show that the levels of platinum accumulation from the drug combinations were greater than the values from LH4-alone. Although the increase in platinum accumulation from the combination of LH4 and CS is not unexpected, when as noteticed both LH4 and CS contribute directly to intracellular platinum pool, the increase in intracellular platinum accumulation from combinations of LH4 and phytochemicals can be seen to indicate that the presence of the phytochemical had served either to increase platinum uptake (and/or to reduce platinum efflux). It is possible that the reduction in oxidative stress by Caps, Cur and Quer somehow enhanced the carrier-mediated transport of LH4 into the cell or its efflux out of the cell and more so in the resistant cell line than in the parent cell line. This was because intracellular accumulation of platinum was generally found to be greater in the resistant A2780^{cisR} cell line than in the parent A2780 cell line. In particular, strongly synergistic

Table II. *Dose-effect parameters applying to combinations of LH4 with Caps, Quer, Cur and CS administered to 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
LH4				4.52	0.47	0.95			10.63	1.27	0.99			7.61	0.47	0.98
Caps				65.05	0.72	0.97			59.07	1.02	0.98			66.18	0.57	0.96
	0/0		0.41	1.38	0.39	0.98		0.48	3.30	0.68	0.99		0.36	1.76	0.36	0.97
LH4 + Caps	0/4	1:4.9	0.52	1.73	0.41	0.98	1:3.04	0.94	6.23	0.90	0.98	1:4.9	0.84	4.08	0.51	0.98
	4/0		0.44	1.49	0.33	0.96		0.79	5.22	0.83	0.99		0.75	3.65	0.43	0.94
Quer				34.80	1.54	0.99			20.17	0.76	0.99			27.05	0.82	0.94
	0/0		0.66	2.07	0.42	0.94		0.31	1.20	0.55	0.98		0.39	1.40	0.31	0.960
LH4 + Quer	0/4	1:2.58	0.64	1.99	0.42	0.88	1:3.46	0.39	1.50	0.58	0.99	1:3.68	0.42	1.53	0.31	0.92
	4/0		0.84	2.62	0.39	0.97		0.69	2.68	0.69	0.99		0.49	1.75	0.31	0.96
Cur				8.88	1.31	0.99			7.82	0.97	0.99			34.76	1.20	0.99
	0/0		0.55	2.13	0.51	0.87		0.55	1.95	0.73	0.95		0.48	1.32	0.55	0.95
LH4+ Cur	0/4	1:1.49	0.55	2.13	0.51	0.87	1:1.3	0.61	2.18	0.64	0.95	1:5.62	0.52	1.42	0.62	0.92
	4/0		0.52	2.01	0.55	0.87		0.37	1.33	0.47	0.98		0.56	1.54	0.53	0.91
CS				1.67	0.92	0.98								9.93	1.18	0.99
	0/0		0.47	2.76	0.24	0.94		0.66	2.90	0.58	0.98		0.91	2.81	0.26	0.99
LH4 + CS	0/4	1:0.14	0.82	4.77	0.37	0.97	1:1.21	0.60	2.64	0.47	0.98	1:1.22	2.40	7.41	0.17	0.99
	4/0		0.41	2.41	0.37	0.93		0.62	2.72	0.57	0.96		0.75	2.30	0.28	0.99

0/0 h and 4/0 h combinations of LH4 with Quer in A2780^{cisR} cell line, and synergistic 0/0 h and 4/0 h combinations of LH4 with CS were found to be associated with high Pt accumulation levels. In contrast, treatments with synergistic 0/0 h combination of LH4 with Cur and strongly synergistic 4/0 h combinations of LH4 with Cur were found to result in relatively lower Pt accumulations in both the parent A2780 and resistant A2780^{cisR} cell lines, although the values in the resistant cell line were slightly greater. The results can be seen also to indicate the complexity of the situation.

Figure 5 depicts the level of platinum–DNA binding in A2780 and A2780^{cisR} cells after their interaction with combinations of LH4 with Caps, Quer, Cur and (at 25 μM concentrations) for 24 h at 37˚C, administered using the 0/0 h and 4/0 h sequences.

The results show that the level of Pt–DNA binding was greater in the resistant A2780^{cisR} cell line than in the parent A2780 cell line from LH4-alone and its combinations with Caps, Quer and CS, in line with higher platinum accumulation resulting from the combinations but not from LH4-alone. However, for the combinations of LH4 with Cur, the level of Pt–DNA binding was greater in the parent A2780 cell line than in the resistant A2780^{cisR} cell line for both 0/0 h and 4/0 h sequences of administration. The results may indicate that, whereas for the combinations of LH4 with Caps, Quer and CS, DNA repair was less significant in the resistant cell than in the parent cell line; for the combination of LH4 with Cur the converse was true.

Mechanism underlying the combined drug action. In attempting to explain the synergism from combinations of LH4 with the phytochemicals, we should note that in the case of Caps and Quer it was the bolus administration the most synergistic; however, it was the pre-treatment with the phytochemicals that resulted in the highest Pt–DNA binding level in the cisplatin-resistant A2780^{cisR} cell line. In contrast, for the combination of LH4 with Cur, although bolus administration resulted in the highest Pt–DNA binding level, it was the pre-treatment with the phytochemical that resulted in most pronounced cell kill.

Although the increased Pt–DNA binding level in the case of Caps and Quer could be (at least in part) a direct result of increased platinum accumulation, in the case of Cur it was more likely to be due to reduced DNA repair (since the increase in platinum accumulation from the presence of Cur was minimal). Irrespective of the mechanisms involved, the observed discordance between Pt–DNA binding level and the degree of synergism can be seen to indicate that the change in combined drug action could not be attributed simply to the change in the Pt–DNA binding level. In this context it is appropriate to note that the phytochemicals (Caps, Quer and Cur), besides acting as anti-oxidants, can themselves induce cell death. For example, Caps was found to inhibit growth and induce apoptosis of various tumour cells including those of leukaemia HL-60 (11), gastric adenocarcinoma (12), breast MCF-7 (12) and glioblastoma U87MG, believed to be mediated *via* p-38 MAPK signalling and mitochondrial

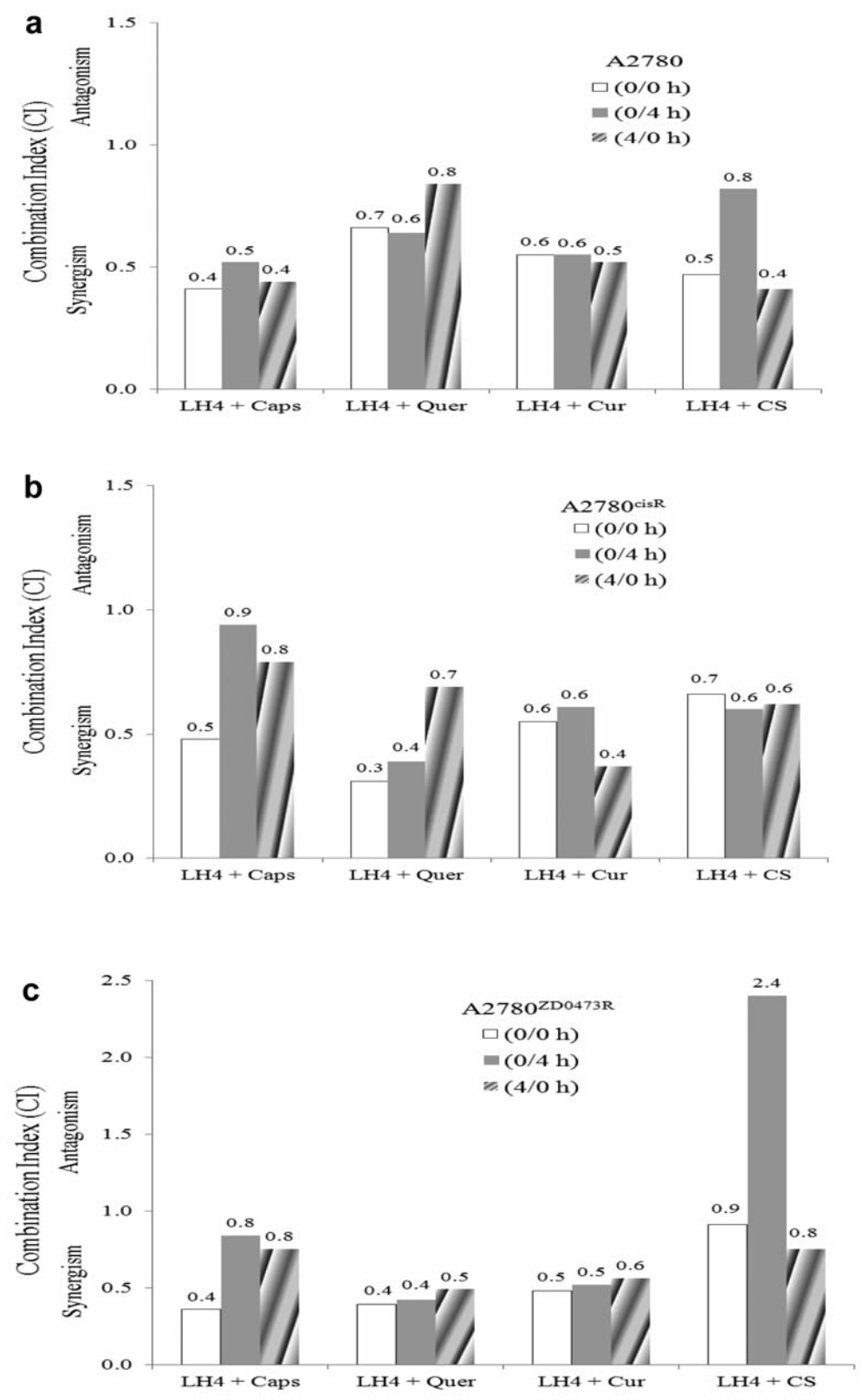

Figure 3. *CIs applying to the 0/0 h, 0/4 h and 4/0 h combinations of LH4 with Caps, Quer, Cur and CS at the Median Effect Dose (ED_{50}) in: (a) A2780 and (b) $A2780^{cisR}$ (c) $A2780^{ZD0473R}$ cell lines.*

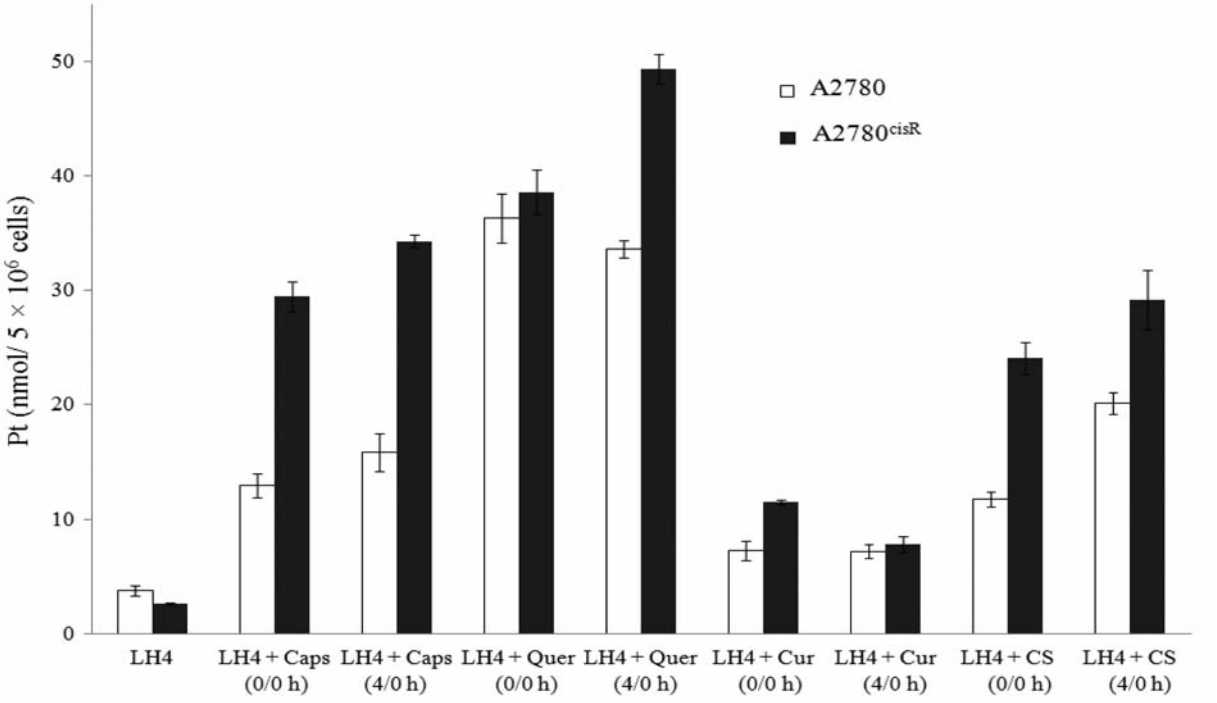

Figure 4. *Platinum accumulations in the A2780 and A2780^{cisR} cell lines resulting from the combinations of LH4 with Caps, Quer, Cur and CS using 0/0 h and 4/0 h sequences of administration after interaction of cells with 25 μM concentrations of compounds for 24 h at 37˚C.*

dysfunction, ROS generation and caspase 3 activation (39). Treatment of cancer cells with Caps was also reported to inhibit the activation of NF-κB by blocking the degradation of the inhibitor of nuclear factor-kappa B alpha (IκBα) and thus preventing the nuclear translocation of the p65 subunit (which is essential for NF-κB activation) (15). Caps was also reported to block the activation of STAT3 (induced by interleukin (IL)-6) (40). JAK1 and c-Src (implicated in STAT3 activation) were with no effect on extracellular signal-regulated kinases (Erk1/2). It was also reported to down-regulate the expression of gene products such as cyclin D1, Bcl-2, Bcl-xL, survivin and vascular endothelial growth factor (VEGF) (40). Finally, whereas Caps induces the accumulation of cells in G_1 phase, monofunctional platinums were found to inhibit proliferation of tumour cells (by arresting cell-cycle progression) at the G_2 phase, thus preventing their mitotic entry and to cause apoptosis through a p53-dependent pathway (38, 41). Thus, it was not unexpected to find that the bolus combination of LH4 with Caps was most synergistic. In this context it is also important to note that the bulky planaramine ligand (benzimidazole) present in LH4 can undergo stacking interaction with base pairs of DNA and can form hydrogen bonds with the DNA. The processes associated with the combination of LH4 and Caps are illustrated in Figure 6 (Aggarwal *et al*., 2008, Zhu *et al*., 2012, Lovejoy *et al*., 2011).

The second phytochemical, Quer, was also reported to inhibit growth of tumor cells (18) and to increase intracellular ROS levels due to production of quercetin radicals (Quercetin-O•) (19), thus, causing free radical-induced apoptosis through ROS/AMPKα1/ASK1/p38 and the AMPKα1/COX2 signalling pathways (20). Quercetin radicals were also found to lower the intracellular GSH pool in a concentration-dependent manner (20) and to trigger apoptosis through mitochondrial depolarization (21). Quer was found to act synergistically in combination with cisplatin in ovarian cancer cells such as OVACA433, CAOV3 and SKOV3 (5), to increase the efficacy of major chemotherapeutic drugs such as doxorubicin and alkylating agents such as dacarbazine (DTIC). It is believed that chemosensitization of doxorubicin by Quer is associated with its ability to down-regulate the expressions of HIF1-a, HER2/neu and/or due to persistent T-cell tumor-specific responses. Both *in vitro* and *in vivo* studies have shown that Quer-mediated down-regulation of HIF1-a, HER2/neu and induction of persistent T-cell tumor-specific responses make cancer cells more sensitive to doxorubicin (42). The ability of Quer to sensitize colon cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and other components of death-inducing signalling complex (DISC) into lipid rafts is believed to be linked to its ability to redistribute TRAIL receptors (43). DTIC is an FDA-approved alkylating agent that is used in the treatment of melanoma (44) but with only a moderate response rate (45).

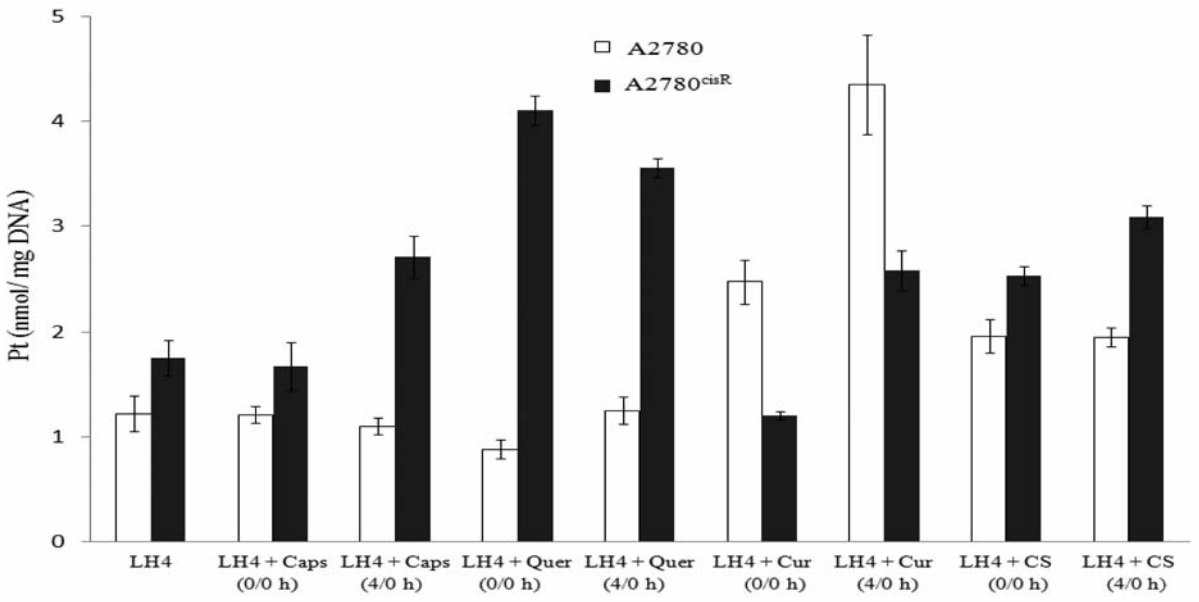

Figure 5. *Pt–DNA binding in the A2780 and A2780^{cisR} cells after their interaction with combinations of LH4 with Caps, Quer, Cur and CS (at 25 μM concentration) for 24 h at 37˚C, administered using the 0/0 h and 4/0 h sequences.*

Treatment with Quer promotes ataxia-telangiectasia mutated (ATM)-dependent phosphorylation of p53 (46) and induces transcriptional activity (47), thereby sensitizing melanoma cells to DTIC. A number of studies have shown that Quer is highly effective as a potent and nontoxic drug that can cause the reversal of multidrug resistance (MDR) in several tumour models (48). It can also sensitize HeLa cells to cisplatin-induced apoptosis through the down-regulation of heat shock protein (Hsp72) and multidrug resistance protein (MRP) (49). The synergistic effect of Quer and CS 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-2cells (50). The third phytochemical Cur is a key component of turmeric with known antioxidant, anti-toxic, anti-inflammatory, cancer chemopreventive and potential chemotherapeutic properties (5, 22). Whereas the resistance to platinum drugs is associated with aberrant activation of NF-κB, the synergistic combinations of LH4 and Cur can dampen its expression, although other mediators are also believed to be involved in Cur-mediated chemosensitization of cisplatin (51). Cur has been found to sensitize ovarian cancer cells (CAOV3 and SKOV3) to cisplatin-induced apoptosis (25, 26) due to inhibition of the Fanconi anemia (FA)/BRCA pathway and production of IL-6 (5). The synergism associated with the combinations of LH4 and CS can also be related to differences in nature of their binding with DNA. Whereas CS is more likely to form intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts with DNA, LH4 can form only monofunctional adducts such as Pt–G) and Pτ–A.

DNA fragmentation. Figure 7 gives the bands observed in the agarose gel electrophoresis of DNAs isolated from A2780 and A2780cisR cells before and after their interaction with selected combinations of LH4 with Caps, Quer, Cur and CS.

Elongation of the DNA band observed in lanes 2-5 in Figure 5 (a) and (b) indicates that interaction of A2780 and A2780^{cisR} cancer cells with LH4 alone and in combination with Caps, Quer, Cur and CS caused damage to DNA. The least intense DNA band resulting from the interaction of A2780 and A2780^{cisR} cancer cells with 0/0 h combination of LH4 and Quer can be seen to indicate the combination that was most damaging to DNA of both A2780 and A2780^{cisR} cancer cells. This is believed to be due to oxidative damage to DNA caused by Quer–O* radical.

Conclusion

Greater activity of designed monofunctional platinum LH4 against resistant tumor models and synergism from combinations with phytochemicals indicate that the compound has the potential for development as a novel platinum-based anticancer drug.

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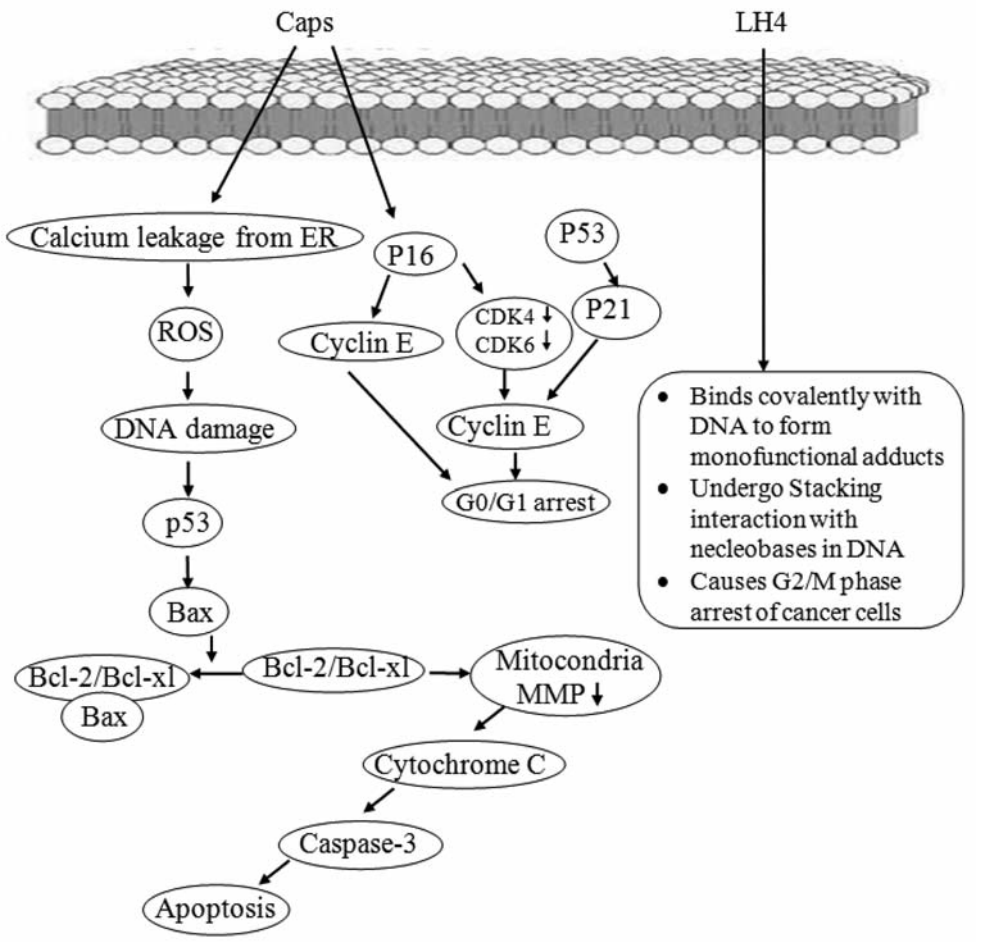

Figure 6. *The suggested processes associated with the synergism activity from combination of LH4 and Caps (15, 41, 38).*

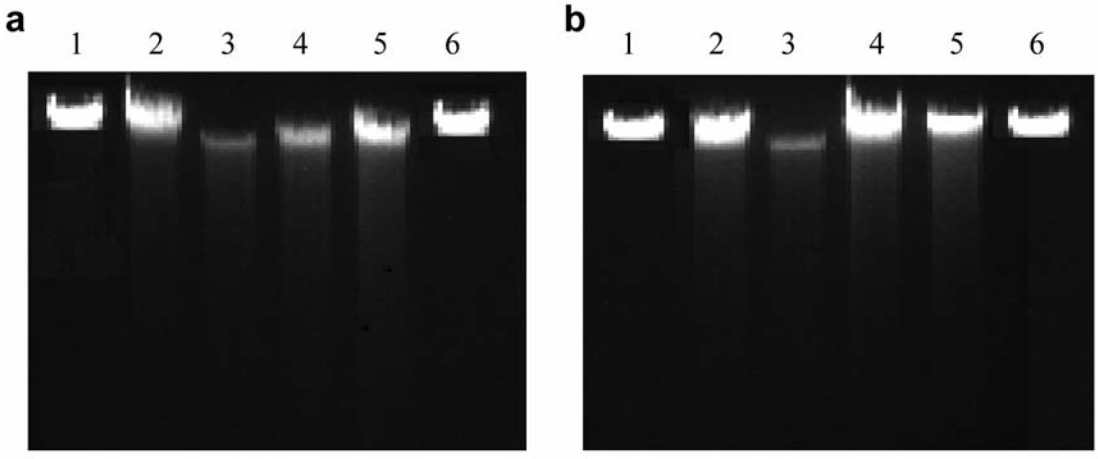

Figure 7. *Agarose gel electrophoresis of DNA from: (a) A2780 cells before and after its interaction with selected combinations of LH4 with Caps, Quer, Cur and CS: Lane 1, Control A2780; Lane 2, LH4 + Caps 4/0 h; Lane 3, LH4 + Quer 0/0 h; Lane 4, LH4 + Cur 4/0 h; Lane 5, LH4 + CS 4/0 h; Lane 6, Control A2780; (b) A2780^{cisR} cells before and after its interaction with selected combinations of LH4 Caps, Quer, Cur and CS: Lane 1, Control A2780^{cisR}; Lane 2, LH4 + Caps 4/0 h; Lane 3, LH4 + Quer 0/0 h; Lane 4, LH4 + Cur 4/0 h; Lane 5, LH4 + CS 4/0 h; lane 6, Control A2780^{cisR}.*

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