Synergism from Sequenced Combinations of Curcumin and Epigallocatechin-3-gallate with Cisplatin in the Killing of Human Ovarian Cancer Cells

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Abstract. *Drug resistance remains an on-going challenge in ovarian cancer chemotherapy. The objective of this study was to determine the effect on synergism in activity from the sequenced combinations of cisplatin (Cis) with curcumin (Cur) and epigallocatechin-3-gallate (EGCG) in the human ovarian cancer cell lines. The drugs were added in binary combinations: Cis combined with Cur, and Cis combined with EGCG to the human ovarian A2780 and A2780^{cisR} cancer cell lines, using five different sequences of administration: 0/0 h, 4/0 h, 0/4 h, 24/0 h and 0/24 h. The combination index (CI) was used to assess the combined action of the drugs. CIs <1, =1 and >1 indicated synergism, additiveness and antagonism respectively. Cellular accumulation of platinum and platinum-DNA binding levels from Cis and its combination with the phytochemicals were determined using graphite furnace atomic absorption spectrometry. Addition of Cis 4 h before Cur and EGCG (0/4 h combination) produced the most synergistic outcomes in both the A2780 and A2780^{cisR} cell lines. The cellular accumulations of platinum and platinum–DNA binding resulting from the 0/4 h combinations were greater as compared to the values using Cis alone, thus providing an explanation for the synergistic action. When sequenced combinations of Cis with Cur and with EGCG are applied to human ovarian A2780 and A2780^{cisR} cancer cell lines, lower concentrations and shorter time gap between the two additions seem to produce a higher cytotoxic effect.*

Ovarian cancer remains the leading cause of death from gynecological cancer in the Western World (1), perhaps due to poor prognosis and the absence of early symptoms (2). Generally, chemotherapy is the main treatment given against advanced ovarian cancer. Despite the presence of numerous side-effects and a limited spectrum of activity due to intrinsic or acquired resistance, the platinum drug cisplatin (Cis) is commonly used to treat various types of cancer including ovarian, lung, bladder, testicular, head and neck and breast cancer, and melanoma (3-5). Combination chemotherapy using two or more drugs having different mechanisms of actions may have a distinct advantage over monotherapy in overcoming drug resistance (6, 7). For example, synergistic combination of Cis with paclitaxel (Tax) has been found to increase life expectancy up to five years for about 50% of ovarian cancer patients (8, 9). Faced with the problems of side-effects and drug resistance, and more importantly having the feeling of helplessness, many cancer patients resort to phytochemicals (such as edible herbs, herbal products, food, drink) along with the targeted therapy hoping to gain an advantage or miracle cure. However, the information on the combined effect of such phytochemicals and the targeted therapy (whether beneficial or harmful) is often lacking. Our own results show that the overall effect from the combination of platinum drugs and phytochemicals as applied to the ovarian tumour models can vary significantly, from being highly synergistic to antagonistic, depending on the sequence of addition. Thus, the possibility remains that cancer suffers may be doing more harm than good when they take phytochemicals along with the targeted therapies.

This study relates to the sequenced combinations of Cis with two phytochemicals curcumin (Cur) and epigallocatechin-3-gallate (EGCG) as applied to the human ovarian tumour models A2780, A2780^{cisR} and A2780^{ZD0473R}. The selected phytochemicals are derived from edible plants and have been consumed by humans for centuries, suggesting that their toxicity may be low or negligible. Cur (from turmeric) and EGCG (from green tea) are consumed in food and drink and are easily available. Their plant sources are cultivated in many parts of the world including China, South

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East Asian countries and the United States. Epidemiological studies suggest that populations that live on a diet rich in Cur have a lower cancer risk (10). Increase in the post-diagnosis consumption of green tea may also boost the survival of patients with epithelial ovarian cancer (11).

Cur has been found to have anticancer properties in various *in vitro* and *in vivo* tumour models (10), including those of pancreatic (12), intestinal and colon (13) rhabdomyosarcoma (14), lung (15) and ovarian (16-18) cancer. It causes cancer cell death through apoptosis (16) and is believed to target multiple molecular targets including pro-apoptotic proteins p53 and BAX, transcription factors NF-κB, Akt and p38 MAPK, and growth factors such as EGF and PDGF (19). Cur is also known to have other medicinal attributes including chemopreventive, antioxidant, anti-inflammatory, antiviral and antibacterial properties (20).

EGCG is the major flavonoid found in green tea (*Camellia sinensis*) green (21). It accounts for 50% to 80% of the total catechin found in green tea (22). Together with other polyphenolic compounds, EGCG makes up about 30% of green tea's dry weight (21). The antioxidant and chemopreventive and anticancer properties of EGCG have been investigated by many (21-25). However, little is known about the effect of combination of Cis with EGCG and Cur. Since the mechanism of actions of Cur and EGCG differ from that of Cis, it is logical to assume that combinations of Cis with Cur and EGCG may display sequence-dependent synergism. In addition, it is appropriate to note that although Cur and EGCG are well-known as antioxidants, they may also act as pro-oxidants at higher concentrations and thus can damage cells (25, 26). Thus, the effects of changes in concentration for the combinations of Cur and EGCG with Cis were also investigated in the present study.

Materials and Methods

Materials. Cis was prepared based on Dhara's method (27). Cur and EGCG in powder form were purchased from SIGMA Aldrich Sydney Australia. Cis was initially dissolved in DMF followed by the addition of mQ water (at a ratio 1:5) to give a 1 mM stock solution. EGCG and Cur were dissolved in ethanol to give a 1 mM stock solution. The drug solutions were serially diluted from the 1 mM stock solutions with freshly prepared RPMI-1640 medium to produce a range of concentrations from 0.5 to 50 μM.

Cell culture. Human ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ were seeded in 25 cm^2 tissue culture flasks in an incubator at 37˚C in a humidified atmosphere consisting of 5% CO_2 in air. The ovarian cancer cell lines were gifts from Ms. Mei Zhang, Royal Prince Alfred Hospital, Sydney, Australia. Stock solutions of platinum compounds (1 mM) were prepared in 1:1 DMF-mQ water mixture and those of plant compounds (1 mM) were made in ethanol. The cells were maintained in logarithmic growth phase in a complete medium consisting of RPMI-1640, 10% heat-inactivated FCS, 20 mM Hepes, 0.11% bicarbonate, and 2 mM glutamine. The cell killing effect due to single and combined treatments was determined using the MTT reduction assay (28). Briefly, 4,000 to 6,000 cells/well in RPMI-1640 medium were seeded into a flat-bottomed 96-well culture plate and allowed to attach overnight. For single compound or drug treatment, drugs were added at a range of at least three to five concentrations to triplicate wells and plates were incubated (37˚C, 5% carbon dioxide in air, pH 7.4) for a period of 72 h. For combination studies, the plant compound and Cis were added using five different sequences namely simultaneous addition (0/0 h), sequential additions using a 4 h gap with Cis added first followed by plant compound (0/4 h) and *vice versa* (4/0 h), and also sequential additions using 24 h gap with Cis added first followed by plant compound (0/24 h) and *vice versa* (24/0 h). For the combination experiments, plant compounds and Cis were added at a constant molar ratio at least three different concentrations that reflected their respective individual IC_{50} values. At the completion of the 72-h incubation period, the MTT assay was performed. The medium was removed and 50 μl of the MTT solution were added to each well of 96-well plate and incubated for four hours. Following this, 150 μl of DMSO were added to each well. The living cells, if still present, remained attached at the bottom stained with MTT as dark blue due to formazan product. The mean absorbance at 570 nm for each compound or drug treatments whether singly or in combination was expressed as a percentage of the untreated control well absorbance. The effect of combining drugs was studied using a median effect analysis whereby a combination index (CI) is calculated from pooled data from 3 to 5 individual experiments each comprising at least three data points for each drug alone and for the drug combinations. The combination index (CI) for two compounds or drugs can be calculated from the following calculation based on Chou and Talalay (29) median effect equation:

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

where D_1 and D_2 are doses of compounds 1 and 2 in combination to achieve x% inhibition, whereas D_{1x} and D_{2x} in the denominator represent doses of compounds 1 and 2 to achieve x% inhibition when present alone. Dx can be readily calculated from the following equation:

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

In the equation, D_x denotes the dose of drug, D_m is the median-effect dose, f_a is the fraction of cells affected (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 (data not shown).

CI values of <1, =1 and >1 indicate respectively synergism, additivity and antagonism in combined drug action. The CI, D_m and r values were obtained automatically using Calcusyn software (V2) (Biosoft, UK). The D_m sometimes reflect the values of IC_{50} value. The linear correlation coefficient, r (where r=1 indicates perfect fit), of the median effect plot should be reasonably good; for the cell culture system, r should be greater than 0.95 (r>0.95) (29).

Pt cellular accumulation. The determination of platinum accumulation in the cells was based on a modification of that described in (30). The platinum and platinum-plant compound combinations (at synergism and antagonism) were added at a constant ratio (1:1) to culture plates containing exponentially growing A2780 and $A2780^{cisR}$ cells in 10 ml of 10% FCS/RPMI-1640 culture medium (cell density of 4,000 to

Table I. *IC_{50} and resistant factor (RF) values for Cis, Cur and EGCG for the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines, where RF is defined as the ratio of the drug concentration required for 50% cell kill when present in combination over that when present alone.*

Drug	IC_{50} (μM)			IC_{50} (μM)		
	A2780	A2780^{cisR}	RF	A2780	A2780^{ZD0473R}	RF
Cis	1.45±0.52	6.64±0.14	4.58	1.45±0.52	4.77±0.46	3.29
Cur	8.39±1.87	6.12±0.77	0.73	8.39±1.87	7.42±1.39	0.88
EGCG	4.46±0.34	5.90±0.81	1.32	4.46±0.34	11.80±0.72	2.65

6,000 cells/0.1 ml). The treatments were conducted in two sets, one for cell uptake studies and another one for platinum and platinum–DNA binding studies. The cells containing the drugs were incubated for 24 h, at the end of which cell monolayers were trypsinized and cell suspension (10 ml) was transferred to a 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. At least three independent experiments were performed. Following incubation with compounds singly and in binary combination, cell pellets were suspended in 0.5 ml of 1% Triton-X, held on ice then sonicated for half an hour. Total intracellular platinum contents were determined by graphite furnace (AAS) using a Varian SpectrAA-20 with a GTA 96 atomic absorption spectrophotometer.

Platinum–DNA binding. Following incubation of cells with single compound or two in combination, high molecular weight DNA was isolated from the cell pellet using JETQUICK Blood DNA Spin Kit/50 (Genomed, Germany) using the modified protocol of Bowtell (31). The cell pellets were resuspended in PBS to a final volume of 200 μl. Following this, 20 μl GENOMED Protease (20 mg/mL), 10 μl of RNase A (20 mg/ml) and 200 μl of buffer K1 (containing guanidine hydrochloride and a detergent) were added to the mixture, which was then incubated for 10 min at 70˚C. After incubation, the sample was mixed thoroughly with 200 μl of absolute ethanol in order to prevent any precipitation of nucleic acids. The sample was then transferred into a JETQUICK micro-spin column containing silica membrane that was positioned inside a 2 ml receiver tube. The sample in the new column was then centrifuged for one minute at 10,600 rpm. The flow through solution in the receiver tube was discarded and the tube was re-attached to its column. The same column was washed with 500 μl of buffer KX (containing high-salt buffer to remove residual contamination) and centrifuged for 1 min at 10,600 rpm. The column was then washed with 500 μl of buffer K2 (containing low-salt buffer to change the high-salt conditions on the silica membrane to low-salt) and centrifuged for 1 min at 10,600 rpm and another one minute at 13,000 rpm to remove any residual liquid. The DNA that binds at the silica membrane of the column was then eluted out in to a new sterile 1.5 ml reaction tube with 200 μl of 10 mM Tris-HCl buffer (pH 8.5). DNA content from each sample was determined by UV spectrophotometry at 260 nm (Varian Cary 1E UV-Visible with Varian Cary Temperature Controller). A_{260}/A_{280} ratio between 1.75 and 1.8 for all samples ensures high purity of DNA (32). The DNA concentration was calculated according to the following equation: concentration=absorbance at 260 nm × 50 ng/μl.

Results

Growth inhibitory effects of single drug. The IC_{50} values of Cis, Cur and EGCG in the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines along with the resistant factors (RFs) are given in Table I. The RF is defined as the ratio of the drug concentration for 50% cell kill when present in combination to that when present alone. The IC_{50} values ranged from 1.45 μM to 6.64 μM for Cis, 6.12 μM to 8.39 μM for Cur and 4.46 μM to 11.80 μM for EGCG. Cis was the most active compound against the parent A2780 cell line whereas Cur was the least active compound. Against the resistant A2780^{cisR} cell line, the most active compound is EGGC, whereas the least active compound is Cis. Against the resistant A2780^{ZD0473R} cell line, the most active compound is Cis, whereas the least active compound was EGCG. Cis was found to have the highest RFs for all the three cell lines, whereas Cur had the lowest RFs. In fact, Cur was found to be more active against the resistant cell lines than the parent cell line, indicating that it was most able to overcome the mechanisms of resistance.

Growth-inhibitory effects of drug combinations. Synergism from combination of Cis with Cur and EGCG was investigated as a function of the sequence of addition in the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines. The effect of changes in concentration on synergism was also investigated for a given sequence of administration.

Sequence of addition. Tables II gives the CIs at ED_{50}, ED_{75} and ED_{90} for the combinations of Cis with Cur and EGCG in the human ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines, where ED_{50}, ED_{75} and ED_{90} is the combined drug concentration required for 50%, 75% and 90% cell kill, respectively.

It can be seen that for the combinations of Cis with Cur and with EGCG in the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines, generally greater synergism was achieved with the 0/4 h sequence of administration than all other sequences. The degree of synergism was extremely high in the 0/4 h combination of Cis with EGCG in the A2780 cell line. Generally the achieved synergism was lower with the 24 h gap of administration than with the 4 h gap. The actual order of synergism from the highest to the lowest was: 0/4 h>4/0 h>0/24 h≅0/0 h>24/0 h. The variations in synergism obtained from sequenced combination of Cis with the two phytochemicals can also be appreciated when we compare D_m values of the compounds when administered alone with and in combination. For example, the D_m values of Cis when combined with Cur and EGCG in the A2780 cell line were 0.21 μM and 0.01 μM respectively. When treated alone, D_m values for Cis, Cur and EGGC in the A2780 cell line were 1.44, 3.78 and 4.36 respectively (Table II). Dose reduction index (DRI), meaning the factor by which the dose or concentration of a drug in combination may be reduced to achieve the same effect

Table II. *Combination indices (CIs) at ED_{50}, ED_{75} and ED_{90} for the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines (denoted as CI_{50}, CI_{75} and CI_{90}).*

Cell line	Drug or drug combination	Sequence	CI_{50}	CI_{75}	CI_{90}	Dm	r
A2780	Cis					1.44	1.00
	Cur					3.78	0.94
	EGCG					4.45	0.98
	Cis+Cur	0/0 h	0.39	0.74	1.46	0.41	1.00
		4/0 h	0.44	0.45	0.48	0.46	1.00
		0/4 h	0.20	0.32	0.53	0.21	1.00
		24/0 h	2.58	1.39	0.78	2.70	0.99
		0/24 h	0.69	0.65	0.64	0.72	1.00
	Cis+EGCG	0/0 h	0.60	0.42	0.37	0.65	0.95
		4/0 h	1.55	1.10	0.99	1.69	0.98
		0/4 h	0.01	0.06	0.58	0.01	0.95
		24/0 h	1.07	0.78	0.71	1.17	1.00
		0/24 h	0.65	0.64	0.80	0.70	0.98
A2780^{cisR}	Cis					5.18	1.00
	Cur					3.56	0.98
	EGCG					4.36	0.99
	Cis+Cur	0/0 h	0.75	0.89	1.05	1.59	1.00
		4/0 h	0.66	0.87	1.16	1.40	0.98
		0/4 h	0.57	0.77	1.06	1.20	0.96
		24/0 h	0.72	1.20	2.00	1.53	0.84
		0/24 h	0.49	0.71	1.03	1.04	0.97
	Cis+EGCG	0/0 h	1.30	1.23	1.17	3.08	1.00
		4/0 h	1.13	1.51	2.01	2.66	0.96
		0/4 h	0.70	1.06	1.60	1.66	0.94
		24/0 h	0.85	1.51	2.68	2.00	0.93
		0/24 h	0.16	0.63	2.51	0.37	0.91
A2780^{ZD0473R}	Cis					4.22	1.00
	Cur					5.14	0.99
	EGCG					6.09	0.95
	Cis+Cur	0/0 h	0.42	0.65	0.99	0.98	1.00
		4/0 h	0.34	0.56	0.92	0.79	1.00
		0/4 h	0.24	0.42	0.73	0.56	0.97
		24/0 h	0.49	0.73	1.10	1.13	0.95
		0/24 h	0.74	2.88	11.24	1.72	0.99
	Cis+EGCG	0/0 h	0.90	0.97	1.04	2.24	0.99
		4/0 h	0.80	0.91	1.05	1.99	0.95
		0/4 h	0.73	0.88	1.06	1.83	0.98
		24/0 h	0.49	0.82	1.37	1.23	0.96

as that obtained from a given concentration of the drug when present alone was also calculated based on Chou and Talaly (29). DRIs are given in Table III.

It can be seen that for the 0/4 h combination of Cis with Cur in the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines, DRI values at the median effect level were 6.75, 4.33 and 7.57 respectively for Cis and 17.68, 2.97 and 9.22 respectively for Cur. The results indicated that in the 0/4 h combination of Cis with Cur, 50% cell kill was obtained with a 6.75-fold reduction in concentration in the case of Cis and 17.68-fold reduction in concentration in the case of Cur. In the A2780^{cisR} cell line, the corresponding reduction in concentration required for 50% cell kill was: 4.33 for Cis and 2.97 for Cur and in the A2780^{ZD0473R} cell line, the values were 7.57 and 9.22 respectively. It should be noted that much greater reduction in concentration required for a given level of activity occurred in the 0/4 h combination of Cis with EGCG, in line with much lower CIs observed for the combination.

Changes in concentration. For the combinations of Cs with Cur and EGCG, the effect of changes in concentration on synergism was investigated, in addition to that of the sequence of addition. Figure 1 shows the effect of changes in concentrations on the CI values applying to the 0/4 h combinations of Cs with Cur and EGCG in the A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines.

Table III. *Dose reduction index (DRI) for the binary combinations of Cis with Cur and EGCG applying to the 0/4 h sequence of administration.*

Cell line	f_a	Drug dose (μM)		DRI		Drug dose (μM)		DRI	
		Cis	Cur	Cis	Cur	Cis	EGCG	Cis	EGCG
A2780	0.50	1.44	9.22	6.75	17.68	1.44	9.22	159.65	491.72
	0.75	14.71	42.53	4.93	8.40	14.71	42.53	30.25	34.83
	0.90	149.98	196.15	3.61	3.99	149.82	196.15	5.73	2.47
$A2780^{cisR}$	0.50	5.18	0.01	4.33	2.97	5.18	0.01	3.12	2.62
	0.75	15.02	0.02	2.88	2.34	15.02	0.02	2.00	1.79
	0.90	43.52	0.07	1.92	1.85	43.52	0.07	1.28	1.22
$A2780^{ZD0473R}$	0.50	4.22	0.01	7.57	9.22	4.22	0.01	2.31	3.33
	0.75	13.21	0.03	4.57	5.00	13.21	0.03	1.97	2.68
	0.90	41.33	0.11	2.76	2.71	41.33	0.11	1.68	2.15

f_a: Fraction of cells killed

For the combinations of Cis with Cur, the CIs were found to increase with increasing concentration in all three cell lines, such that at very high concentrations, the combined drug action was actually antagonistic rather than being synergistic. For the combinations of Cis with EGCG also, the CIs were found to increase with increasing concentration in the two resistant $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines. However, in the parent A2780 cell line, the CIs were essentially independent of changes in concentration except at very high concentrations.

Cellular platinum accumulation and platinum–DNA binding. Since the 0/4 h combination of Cis with Cur and EGCG showed the greatest synergism, whereas the 24/0 combination showed the greatest antagonism, these two sequences were selected for the determination of cellular accumulation of platinum and platinum–DNA binding levels in the A2780 and $A2780^{cisR}$ cell lines. The aim was to determine whether there was any correlation between the degree of synergism and the values for platinum accumulation and platinum–DNA binding.

It can be seen that the platinum accumulation from the synergistic combinations in both the A2780 and $A2780^{cisR}$ cell lines was generally greater than those from the antagonistic combinations. Accumulation from the synergistic combinations was also greater than that from Cis alone. In fact, the values found with Cis alone were also lower than those from the antagonistic combinations. The greatest difference in platinum accumulation from the synergistic and antagonistic combinations was observed for the combinations of Cis with EGCG in the A2780 cell line (Figure 2). The other point to note is that generally, platinum accumulation in the parent A2780 cell line was greater than that in the resistant $A2780^{cisR}$ cell line.

Comparing platinum–DNA binding levels resulting from the synergistic combinations of Cis with EGCG and with Cur with the corresponding values from the antagonistic combinations, it can be seen that the former were greater than the latter in the case of Cis plus EGCG by 4.1-fold in the A2780 cell line and 1.2-fold in the $A2780^{cisR}$ cell line, in the case of Cis plus Cur: 2.0 times greater in the A2780 cell line and 1.5 times greater in the $A2780^{cisR}$ cell line. The highest platinum–DNA binding level observed for the synergistic combination of Cis with EGCG can be seen to be in agreement with the highest level of platinum accumulation resulting from the same combination.

Discussion

The lower IC_{50} values for Cur in the resistant $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines than in the parent A2780 cell line indicate that the compound has been able to overcome the mechanisms of resistance operating in the resistant cell lines. Other investigators also reported that Cur showed greater activity in the cisplatin-resistant cell lines than in the cisplatin-sensitive ones (32). Although our knowledge on the exact mechanisms of resistance in the ovarian cancer cell lines remains incomplete, it is believed that platinum resistance in the $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines is associated with one or more of the following: (i) reduced cellular accumulation of platinum, (ii) increased deactivation of the drug before binding with DNA, (iii) increased DNA repair and (iv) increased tolerance of platinum–DNA adducts (33). Since Cur is not a DNA binder, the increased activity of Cur in combination therapy in the resistant cell lines point to the fact that although apoptosis due to platinum drugs may be a consequence of platinum–DNA binding, it is not sufficient on its own in these cells to bring about the cell

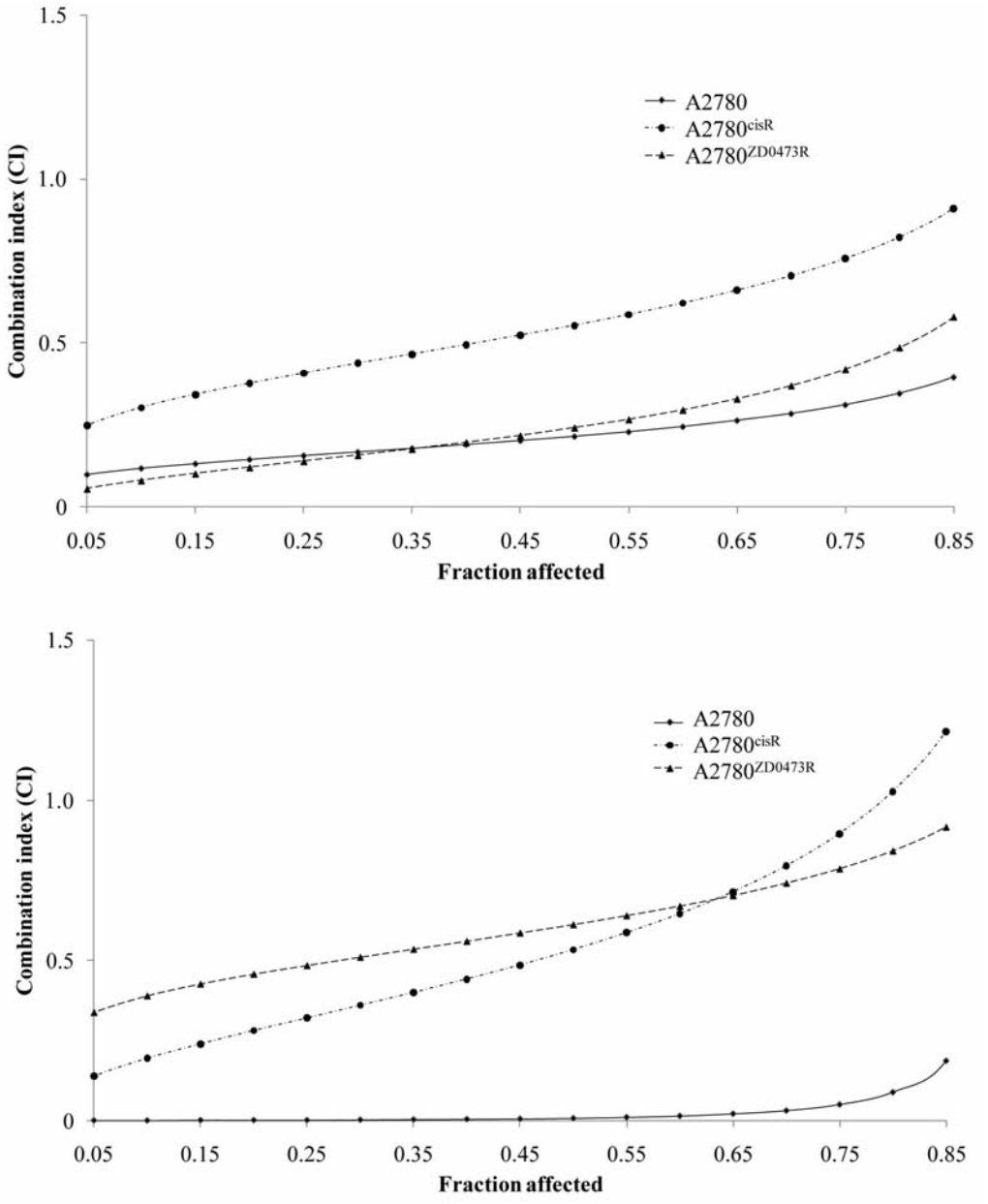

Figure 1. *CI versus affected cell-fraction (fa) curves for the 0/4 h combination of (a) Cis with (a) Cur and (b) with EGCG.*

death. Rather it may trigger a series of reactions that ultimately bring about cell death. It is possible that the differential expressions of one or more proteins associated with platinum resistance in the ovarian cancer cell lines, although deactivating as applied to the action of platinum, serve to enhance apoptosis due to Cur.

As applied to the synergism resulting from the combination of Cis with Cur and with EGCG, the most notable finding of the present study is that the greatest synergism was achieved when Cis was added first followed by the phytochemical 4 h later, and generally the least synergism (often antagonism) was achieved when the phytochemical was added first followed by Cis 24 h later. The other important finding of this study is that it confirms the work of Montopoli *et al*. who reported that when Cis was combined with Cur, greater synergism was achieved at much lower concentrations than at higher concentrations (33).

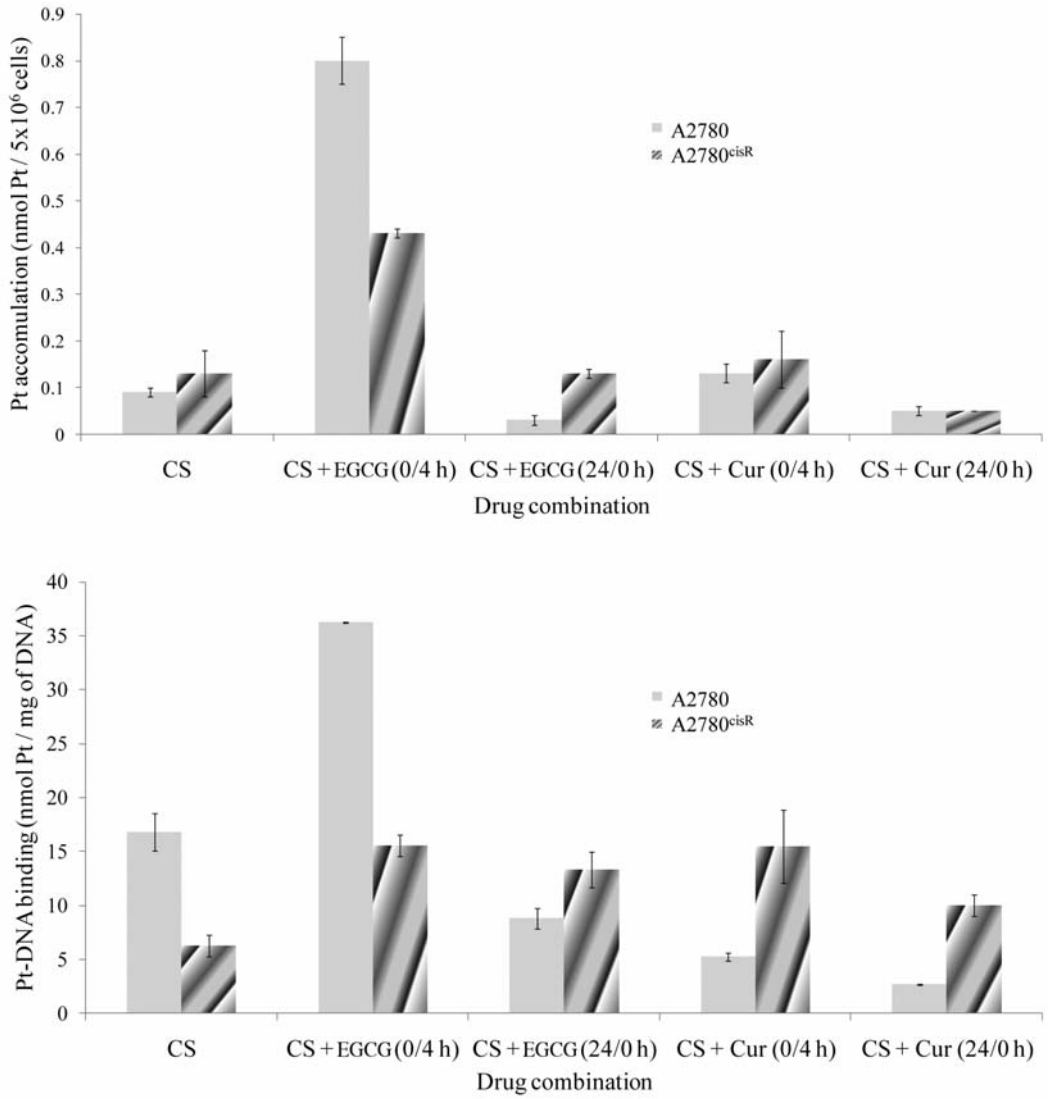

Figure 2. *Platinum accumulation (a) and platinum–DNA binding level (b) in the A2780 associated with 0/4 h and 24/0 h combinations.*

The highest platinum–DNA binding level, resulting from the synergistic combination of Cis and EGCG, was in line with the level of intracellular accumulation of platinum for this combination being highest, and the lowest level of platinum–DNA binding observed from the antagonistic combination of Cis and Cur in the A2780 cell line was also in line with low level of intracellular platinum accumulation from the combination. The results can be seen to support the idea that one of the factors responsible for synergism from the combinations of Cis with EGCG and Cur (especially EGCG) is associated with the enhancement of cisplatin action. The increased platinum-DNA binding may serve to dampen the effect of DNA repair which is believed to be a dominant mechanism of platinum resistance in the ovarian cancer (34).

The high synergism resulting from the 0/4 h combination of Cis with EGCG means that when administered in that sequence, the cell killing effect was achieved at much lower concentrations as compared to the concentration required when present alone. It was reported that EGCG amplified the cytotoxicity of Cis by 3- to 6-fold in ovarian cancer cells (15, 36). It has been suggested that EGCG may accentuate the oxidative stress that would serve to inhibit the growth of ovarian cancer cells and sensitize them to the action by Cis. It has been further suggested that the cytotoxic effect of Cis was closely associated with the increased generation of reactive oxygen species (ROS) (37). Once the ROS level is elevated, this would serve to reduce the level of intracellular thiols [such as glutathione (GSH) which is the main

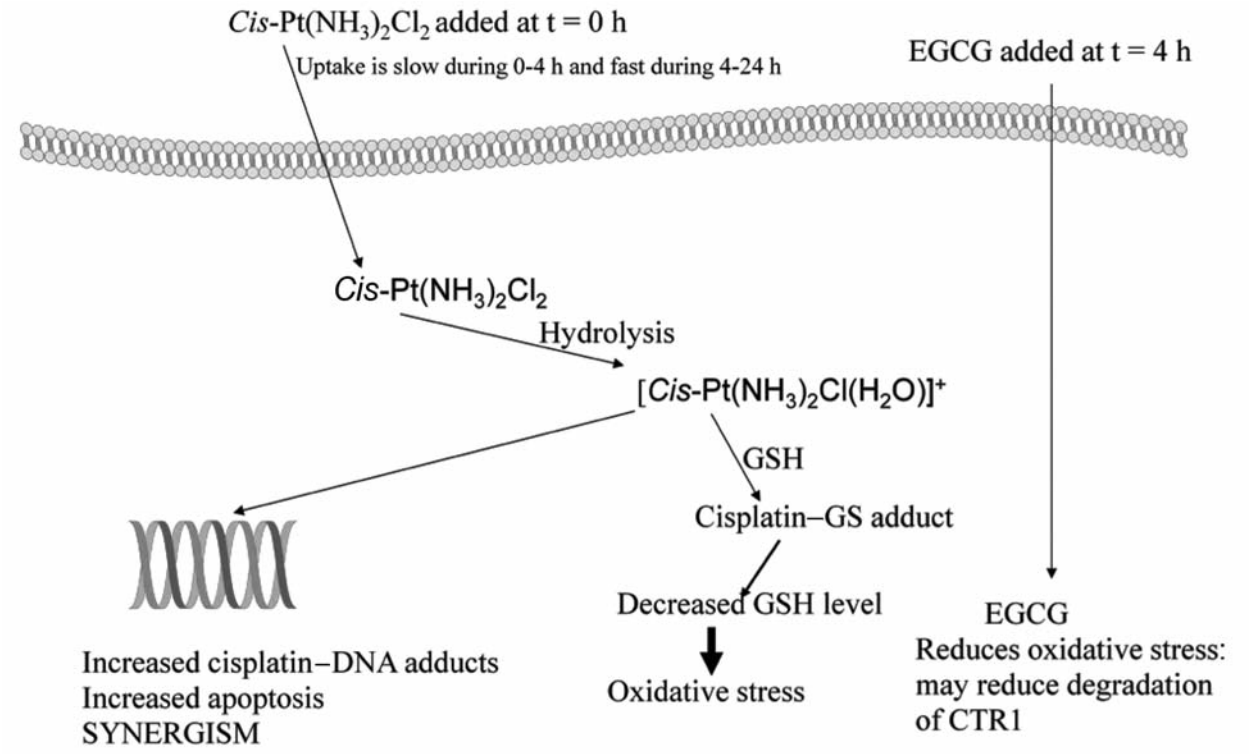

Figure 3. *Suggested processes associated with the activity of the 0/4 h combination of Cis and EGCG: During 0-4 h, Cis enters cells by passive diffusion and carrier-mediated transport. Once inside the cell, it undergoes hydrolysis and binds with cellular thiols such as glutathione (GSH) and DNA. Formation of Cis–glutathione adducts reduce cellular GSH level producing oxidative stress. EGCG added at 4 h reduces oxidative stress and prevents proteasome-induced degradation of the carrier CTR1, thus resulting in increased availability of Cis.*

intracellular antioxidant in the body] that are involved in detoxification of ROS. As the level of intracellular thiols decreases, less platinum will be deactivated resulting in increased binding of platinum with DNA. It is thus hypothesised that the addition of Cis first, namely 4 h before the addition of Cur and EGCG, to the A2780^{cisR} cancer cells will serve to increase the activity of ROS in the cells. As noted earlier, EGCG is a polyphenolic compound found in green tea that has been shown to provide protection against DNA damage by acting as a scavenger for free radicals (38). As to the question of why the addition of Cis 4 h before the addition of EGCG greatly increased the cellular accumulation of Cis in both the A2780 and A2780^{cisR} cell lines (Figure 2), it is hypothesized that in some respect, EGCG may also be acting like a proteasome inhibitor such as bortezomib. Bortezomib has been found to retard the Cis-induced down-regulation of copper transporter hTR1 that is involved in transporting Cis into the cell (39). This idea is illustrated in Figure 3.

Conversely, the antagonism resulting from the addition of the plant compounds 4 h before the administration of Cis can also be related to their antioxidant activity, which serves to decrease the ROS level. A decrease in the ROS level would spare cellular thiols from deactivation (by ROS), resulting in increased platinum–thiols binding and therefore reduced platinum–DNA binding. Recently, a 67-kDa lamin receptor (67LR) has been identified as a cell surface receptor for EGCG that is believed to mediate antitumour activity of EGCG (40). 67LR originates from the ribosomal protein p40, which is a component of the translational machinery of the cell. Umeda *et al*. suggest that EGCG-induced inhibition of tumour growth is abrogated by the silencing of 67LR, eukaryotic translation factor 1A (eFFIA) or 110-130-kDa myosin phosphatase-targeting subunit (MYPT1) in tumour cells (40).

In considering the above results, it is important to note that the addition of Cis first followed by phytochemical 4 or 24 h later is expected to increase both the cellular accumulation of platinum and the platinum–DNA binding level (due to longer period of incubation), thus providing a further explanation as to why the platinum–DNA binding level from the synergistic 0/4 combination of Cis and Cur was almost twice that of the antagonistic 24/0 h combination. However, the period of incubation alone cannot explain why the platinum–DNA binding level from Cis alone was significantly less than that from the 0/4 h combination of Cis and EGCG. Similarly, the difference in the period of exposure to Cis cannot explain why

platinum–DNA binding levels from the 4/0 h combination of Cis with Tax in both the A2780 and A2780^{cisR} cell lines were found to be greater than those from Cis alone (42). The results can be seen to indicate that drug–drug interaction must be playing key roles in modulating both the transport of platinum across the cell membrane, as well as the level of binding of platinum with DNA.

When we compare platinum–DNA binding levels in the A2780 and A2780^{cisR} cell lines from 0/4 h combination of Cis and EGCG with those from Cis alone, it is found that the values from the combination are higher than those from Cis alone. Moreover, the level in the resistant cell line is lower than that in the parent cell line (although as noted earlier, there was no significant difference in the cellular accumulation of platinum). The results can be seen to provide support to the idea that increased DNA repair and/or increased deactivation of the platinum drug within the cell (due to its increased binding with cellular thiol such as GSH) may be a dominant mechanism of resistance in the resistant cell line A2780cisR. If this is so, EGCG may be playing two contrasting roles: (i) by acting as a free radical scavenger, it serves to protect cancer cells rather than causing cell death; (ii) by enhancing the action of Cis, it serves to promote cell death. This hypothesis may explain why the 24/0 h combination of Cis and EGCG is least synergistic, whereas the 0/0 h and 0/4 h combinations (especially 0/4 h) are highly synergistic. Although the duration of exposure of cancer cells to Cis would be the same in both 0/0 h and 0/4 h additions, in the former, any effect of EGCG would be absent during the first four hours. As to the question of how EGCG can bring about death of cancer cells, it is important to note that antioxidants are well known to act as pro-oxidants at higher concentrations (15, 26). It has been reported that although the basal ROS level was lower, the GSH level was significantly higher in cisplatin-resistant cell lines than in cisplatin-sensitive ones (35). Cur was found to reduce the GSH level after 2 h incubation but increase it after 24 h in both the resistant and sensitive cell lines. Other findings also support the early pro-oxidative effect (increased ROS and reduced GSH level) and the late antioxidant effect (increased GSH level) of Cur in leukemia cells and ovarian cancer cells (18, 43). It is well known that an increase in the cellular levels of ROS, such as H_2O_2, results in the activation of NF-kB and many reports have shown that Cur can reduce the cellular levels of ROS *in vivo*. It is suggested that Cur may prevent the activation of NF-kB *in vivo* by reducing cellular ROS levels.

The results can be seen to support the idea that one of the factors responsible for synergism from combinations of Cis with EGCG and with Cur is associated with the enhancement of Cis action. It was reported that Cur in the micromolar range (> 15 μM) caused DNA damage as indicated by the Comet assay in human gastric mucosal cells and peripheral blood lymphocytes (43).

In conclusion, as applied to sequenced combinations of Cis with EGCG and with Cur, lower concentrations and shorter time gap between the two additions seem to produce a higher cytotoxic effect. If this is also true *in vivo*, such combinations may provide a means of overcoming drug resistance. Thus, *in vivo* studies using suitable animal model are warranted to ascertain whether the results of the present study apply *in vivo* or not. Further studies on proteomics are also needed to obtain more complete information about proteins involved in drug resistance. It would also be important to determine platinum accumulation and Pt–DNA binding levels associated with combinations that show lower synergism or even antagonism, as these results may provide further light on the processes taking place at the epigenomic level. Proteomic studies may be supplemented with microarray analyses to provide further information at the genomic level.

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