Synergism from the Combination of Oxaliplatin with Selected Phytochemicals in Human Ovarian Cancer Cell Lines

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Abstract. *Oxaliplatin (Oxa) is a third generation platinum drug currently in clinical use for the treatment against colorectal cancer. Although it has a somewhat different spectrum of activity than cisplatin (Cis), it too has two major limitations, namely problems of side-effects and drug resistance. In this study, combined drug action from the combination of Oxa with the phytochemicals andrographolide (Andro), epigallocatechin-3-gallate (EGCG), chlorophyllin (Chl), colchicines (Col), curcumin (Cur) and paclitaxel (Tax) was evaluated in the human ovarian cancer cell lines A2780 and A2780^{cisR}. The combination index (CI) was used as a measure of synergism (CI<1), addictiveness (CI=1) and antagonism (CI>1). Generally, all the combinations showed greater synergism at a lower concentration (ED_{50}) than at higher concentrations (ED_{75} and ED_{90}). Oxa in binary combination with Col and Tax showed the highest synergism in both the cancer cell lines, when administered 4 h after the phytochemical, with CI at ED_{50} ranging from 0.004 to 0.1. The combination of Oxa with the other phytochemicals generally showed greater synergism when Oxa was administered 4 h before the phytochemical. Appropriately sequenced combination of Oxa with tumor active phytochemicals produces marked synergistic effects in cisplatin resistant as well as non-resistant ovarian cancer cell lines and may offer the means of overcoming drug resistance in ovarian cancer.*

Oxaliplatin (Oxa), a third generation platinum drug approved by the Food & Drug Administration (FDA) in the US in 2002 for the treatment of colorectal cancer, is a cisplatin (Cis) analogue in which the carrier ligand is 1,2-diamminocyclohexane (DACH) as against two ammonia ligands in Cis (1). Oxa is currently used clinically alone and in combination with other drugs in advanced stage disease (2). Like other platinum drugs, Oxa works better in combination with non-platinum drugs such as 5-fluorouracil (5-FU) and leucovorin (LV) (3) although even in such combinations it causes some side-effects (including persistent peripheral neuropathy, nausea and vomiting) (4). It is generally believed that the combination of drugs with different mechanisms of action may offer the means of overcoming drug resistance and reducing the side-effects (5).

Approximately 67% of all anticancer drugs originate from plants with the bioactive compounds belonging mainly to alkaloid, phenolic and terpenoid families (6). Although cancer patients use herbal products along with targeted therapy (hoping to gain miracle cure or advantage) very little is known about the combined effects (beneficial or adverse). In the present study, binary combinations of a number of phytochemicals namely andrographolide (Andro), curcumin (Cur) and epigallocatechin-3-gallate (EGCG) that are known to be present in the respective herbs *Andrographis paniculata* (known as hempedu bumi in Malaysia), *Curcuma longa* (turmeric) and *Camellia sinensis* (green tea) and chlorophyllin (Chl) a derivative of chlorophyll with Oxa, were tested for cytotoxicity against human ovarian cancer cell lines. These herbs are believed to be safe as they are commonly consumed as drink, food or food supplements. Data from a phase I clinical trial showed that Cur is non-toxic up to a dose of 8000 mg/daily (7). Combinations of paclitaxel (Tax) and colchicine (Col) with Oxa were also studied for comparison. This study constitutes a part of our continued studies on combinations of platinum drugs with phytochemicals in human ovarian tumour models (8, 9).

Materials and Methods

Materials. Oxa, Andro, EGCG, Chl, Col, Cur and Tax in the powder form were obtained from SIGMA-Aldrich (Sydney, Australia). Oxa was initially dissolved in dimethylformamide (DMF) followed by dilution with milli Q (mQ) water (at a ratio 1:5) to produce a 1 mM stock solution. Each of the phytochemicals was dissolved in ethanol

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to give a 1 mM stock solution. The drug solutions were serially diluted from the 1 mM stock solutions with freshly prepared RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) to prepare a range of final concentrations from 0.0005 to 100 μM.

Cell culture. Human ovarian cancer cell lines A2780 (parent) and A2780^{cisR} (Cis-resistant) were obtained from Ms. Zhang from the Royal Prince Alfred Hospital, University of Sydney, Australia. The cell lines were sub-cultured in RPMI 1640 medium that was prepared in 10% FCS, 1 mM Hepes, 5.6% sodium bicarbonate and 200 mM glutamine. The cell kill was determined using the MTT reduction assay (10). Briefly, 4000 to 5500 cells per well in RPMI-1640 medium were seeded into a flat-bottomed 96-well culture plate and allowed to attach overnight. For single drug treatment, the solutions were added at a range of at least three to five concentrations to triplicate wells and left in the incubator (37˚C, 5% carbon dioxide in air, pH7.4) for 72 h. For combination studies, binary combinations of Oxa with the phytochemicals at a constant ratio (based on IC_{50} values) were added using five different sequences: simultaneous addition (0/0 h), sequential additions with 4 h time gap with Oxa added first followed by the phytochemical (0/4 h) and vice versa (4/0 h), and also sequential additions using 24 h time gap with Oxa added first followed by plant compound (0/24 h) and the converse (24/0 h). After 72 h of incubation, the MTT assay was performed as described earlier (10). The combined effect was studied using the median effect analysis; the combination index (CI) was calculated using pooled data from 3 to 5 individual experiments each comprising at least three data points. The calculation of CI for two drugs in combination was based on the median effect equation of Chou (11).

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

where D_1 and D_2 in the numerator stand for the concentrations of compounds 1 and 2 in combination to achieve x% inhibition whereas D_{1x} and D_{2x} in the denominator represent concentrations of compounds 1 and 2 to achieve x% inhibition when present alone. D_x can be readily calculated from the following equation. In the equation D_x denotes 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).

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

CI of <1, = 1 and >1 indicates respectively synergism, additivity and antagonism in combined drug action. The CI, D_m and r values were calculated using Calcusyn software (V2) (Biosoft, Cambridge, UK). D_m means median effect dose which sometimes reflects the values of IC_{50}. The linear correlation coefficient, 'r' indicates the goodness of fit for the pooled data (where r=1 is a perfect fit).The r value of the median effect plot for the cell culture system should be greater than 0.95 (r>0.95) (14)

Results

Growth inhibitory effects of single drugs. The IC_{50} values of Oxa, Tax, Col, EGCG, Chl, Cur and Andro in the A2780 and A2780^{cisR} cells along with the resistant factors (RFs) are provided in Table I. The resistant factor RF is defined as the ratio of the drug concentration required for 50% cell kill in the resistant cell line to the concentration required in the parent cell line.

Table I. *IC_{50} and resistant factor (RF) values of the platinum-based drugs and phytochemicals in the human ovarian A2780 and A2780^{cisR} cancer cell lines.*

Drug	A2780	A2780^{cisR}	RF
Cis	1.45±0.52	6.64±0.14	4.58
Oxa	0.55±0.04	0.97±0.10	1.76
Andro	19.69±1.10	6.66±1.66	0.34
EGCG	4.46±0.34	5.90±0.81	1.32
Chl	7.09±0.43	5.00±0.56	0.71
Col	0.02±0.006	0.02±0.013	1.00
Cur	8.39±1.87	6.12±0.77	0.73
Tax	0.003±0.002	0.006±0.0002	2.00

The order of cytotoxicity from the highest to the lowest in the A2780 cell line was Tax>Col>Oxa>Cis>EGCG>Chl>Cur>Andro. The drugs with higher cytotoxicity Tax, Col, Oxa and Cis were found to have RFs >1 indicating that the compounds had lower activity in the resistant cell line than the parent cell line. In contrast, the less active compounds Andro, Cur and Chl were found to have a higher activity in the resistant A2780^{cisR} cell line than the parent cell line.

Growth inhibitory effects of drugs in combination. Figure 1 provides the CI values at ED_{50}, ED_{75} and ED_{90} applying to combinations of Oxa with the phytochemicals in the A2780 cell line and Figure 2 provides the same as applied to the A2780^{cisR} cell line. ED_{50}, ED_{75} and ED_{90} stand for 50%, 75% and 90% cell kill respectively and CI_{50}, CI_{75} and CI_{90} indicate the corresponding CI values. Table II provides the D_m, m and r values.

Generally, greater synergism was observed when Oxa was administered 4 h before the phytochemical and the degree of synergism was greater at the lower concentration (ED_{50}) than at higher concentrations (ED_{75} and ED_{90}).

For the combination of Oxa with Col, marked synergism was also produced when the drugs were administered using the 24/0 h sequence (Figure 2).

Only a few combinations showed a higher level of synergism in the A2780^{cisR} cells as compared to the one in the parent A2780 cells. These included the 0/0 h combination of Oxa with Cur, Col and EGCG, 0/4 h combinations of Oxa with Andro, Col, Chl and EGCG, and 4/0 h combination of Oxa with EGCG (Figure 3).

For combinations of Oxa with Col and Tax, greater synergism was produced in the A2780 than in A2780^{cisR} cells although the degree of synergism in both cell lines was very marked (CI<0.5) (particularly when administered using

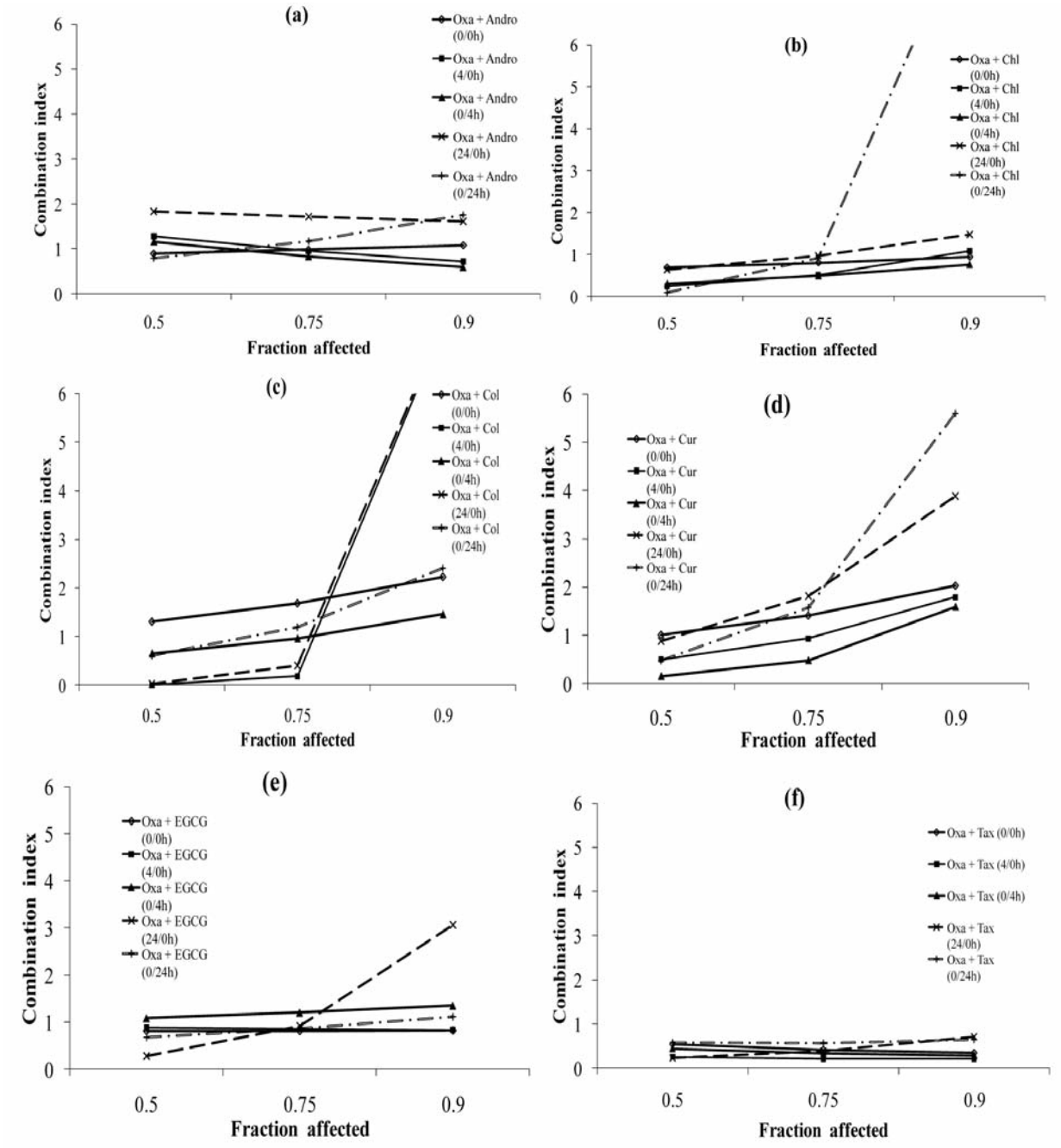

Figure 1. *CIs of oxaliplati with andrographolide (a), chlorophyllin (b), colchicine (c), curcumin (d), epigallocatechin-3-gallate (e) and paclitaxel (f) using five different sequences of administration of oxaliplatin/phytochemical (when 0h was time of administration of first drug) in A2780 cells, ED: effective dose, % cell kill.*

particularly the 4/0 h and 24/0 h sequences). For the other drug combinations, higher synergisms were shown when they were administered using the sequence 0/4 h (Figure 3); Oxa with Cur and Chl showed strong synergism and with Chl the degree of synergism was greater in the A2780^{cisR}, as compared to the one in the A2780 cells. The combination of Oxa and Andro showed the least synergism in both cell lines.

Discussion

The combinations of Oxa with the selected phytochemicals produced both sequence- and concentration-dependent synergism in the human ovarian A2780 and A2780^{cisR} cells. At ED50, high synergism was produced from the 0/4 h combination of Oxa with Cur and the 0/24 h combination of

Table II. *Dose effect parameters of oxaliplatin and phytochemicals using five different sequences of administration: oxaliplatin/phytochemical (when 0h was of administration of first drug) in A2780 and A2780^{cisR} cells.*

Time sequence	Drug (s)	A2780			A2780^{cisR}			Drug (s)	A2780			A2780^{cisR}		
		D_m	m	r	D_m	m	r		D_m	m	r	D_m	m	r
	Oxa	0.64	0.76	1.00	2.45	0.93	0.94							
	Andro	14.68	0.66	0.99	8.17	1.01	1.00	Cur	3.74	0.59	0.94	3.56	0.89	0.98
0/0 h	Oxa	0.40	0.69	0.97	0.61	1.08	0.98	Oxa	0.35	0.56	0.92	0.40	0.68	0.96
4/0 h	+ Andro	0.57	0.90	1.00	0.70	0.98	0.96	+ Cur	0.17	0.49	0.96	0.45	0.72	0.96
0/4 h		0.52	0.93	0.99	0.55	1.00	0.97		0.05	0.39	0.99	0.27	0.77	0.96
24/0 h		0.82	0.76	0.96	0.58	1.03	0.97		0.30	0.47	1.00	0.40	0.88	0.98
0/24 h		0.35	0.57	0.99	0.48	0.90	0.98		0.16	0.39	0.99	0.37	0.64	0.98
	Chl	9.23	0.70	1.00	4.70	0.87	1.00	Col	0.01	0.96	1.00	0.01	0.91	0.99
0/0 h	Oxa	0.26	0.66	0.99	0.40	0.66	1.00	Oxa	0.57	0.69	0.98	0.61	1.05	1.00
4/0 h	+	0.09	0.49	1.00	0.41	0.65	0.96	+	0.00	0.22	0.99	0.08	0.51	0.92
0/4 h	Chl	0.11	0.56	0.99	0.10	0.45	0.98	Col	0.28	0.63	1.00	0.44	0.85	0.98
24/0 h		0.24	0.57	1.00	0.86	0.50	0.89		0.01	0.26	1.00	0.10	0.32	0.91
0/24 h		0.03	0.29	0.99	0.21	0.32	0.95		0.26	0.54	0.98	0.86	0.45	1.00
	EGCG	4.45	0.82	0.98	4.27	0.99	0.99	Tax	0.01	0.44	0.91	0.01	0.97	0.99
0/0 h	Oxa	0.30	0.78	0.99	0.37	0.91	1.00	Oxa	0.26	0.81	0.97	0.88	0.93	1.00
4/0 h	+	0.33	0.80	0.95	0.31	0.93	0.99	+	0.12	0.73	0.96	0.29	0.62	0.99
0/4 h	EGCG	0.40	0.72	0.97	0.35	0.70	0.99	Tax	0.21	0.81	0.99	0.66	0.75	1.00
24/0 h		0.10	0.42	1.00	0.50	0.82	1.00		0.11	0.51	0.96	0.43	0.66	1.00
0/24 h		0.25	0.66	0.91	0.56	0.59	0.98		0.27	0.67	0.97	0.67	0.72	0.98

Oxa with Chl in the A2780 cell line, and the 4/0 h combination of Oxa with Tax, and both 4/0 h and 24/0 h combinations of Oxa with Col in both A2780 and A2780^{cisR} cells. The results suggested that the phytochemicals differ in the mechanisms of their antitumor action. Tax is a microtubule inhibitor that promotes polymerization of tubulin. Cur is believed to target multiple molecular targets including pro-apoptotic proteins p53 and BAX, transcription factors NFκB, Akt and p38 map kinase (p38 *MAPK*), and growth factors such as EGF and PDGF. Cur is also known to have other medicinal attributes including chemopreventive, antioxidant, anti-inflammatory, antiviral and antibacterial properties.

Oxa on its own is more active than Cis in both A2780 and A2780^{cisR} cell lines (9). The first evidence for the lack of cross-resistance between Oxa and Cis or its analogue carboplatin in A2780 and cisplatin-resistant A2780^{cisR} cell lines, was reported by Pendyala and Creaven (12). Oxa also being a Cis analogue (possessing two labile and two non-labile ligands in a cis-geometry) is expected to bind with DNA forming mainly intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts (in addition to monofunctional adducts) that would cause more of a local bending of a DNA strand. However, since Oxa contains bulky DACH carrier ligand, it forms a smaller number of adducts than Cis and is bound to differ from Cis in terms of non-covalent interactions with the DNA (13, 14).

Although the exact mechanisms of platinum resistance remain incompletely understood, often they are attributed to decreased drug accumulation, increased expression of intracellular thiols, altered expression of regulatory genes and increased DNA repair activities (15).

In addition the adducts of Oxa with DNA are not recognized by mismatch repair (MMR) complex (that helps in repairing DNA), but MMR recognizes Cis-adducts, thus explaining why Oxa lacks cross resistance with Cis (16).

Synergism from the combinations of Oxa with phytochemicals such as taxanes (*e.g.* paclitaxel) and topoisomerase inhibitors (*e.g.* irinotecan and topotecan) has been reported previously (16). The present studies demonstrated that combinations of Oxa with Tax or Col using both 0/4 and 4/0 h sequences of administration produced greater synergism than the bolus administration. Administration of Tax followed by Oxa could be more useful clinically due to longer persistence of Oxa in the systemic circulation (4). In our previous study (9) greater synergism was produced from the 4/0 h sequence of administration of Cis with Tax than 0/4 h and 0/0 h sequences. This was an expected result, as Tax blocks progression in the cell cycle in an earlier phase than Cis (G_2/M in the case of Tax and G_1/S in the case of Cis) (17). The same explanation could be attributed to the combinations of Oxa with Tax and Col.

In this study the combinations of Oxa with Cur, Chl and EGCG, produced greater synergism when the phytochemicals were added 4 h later (0/4 h) than the converse (4/0 h). Not many studies on the effects of combinations of Oxa with Andro, Cur, Chl and EGCG have

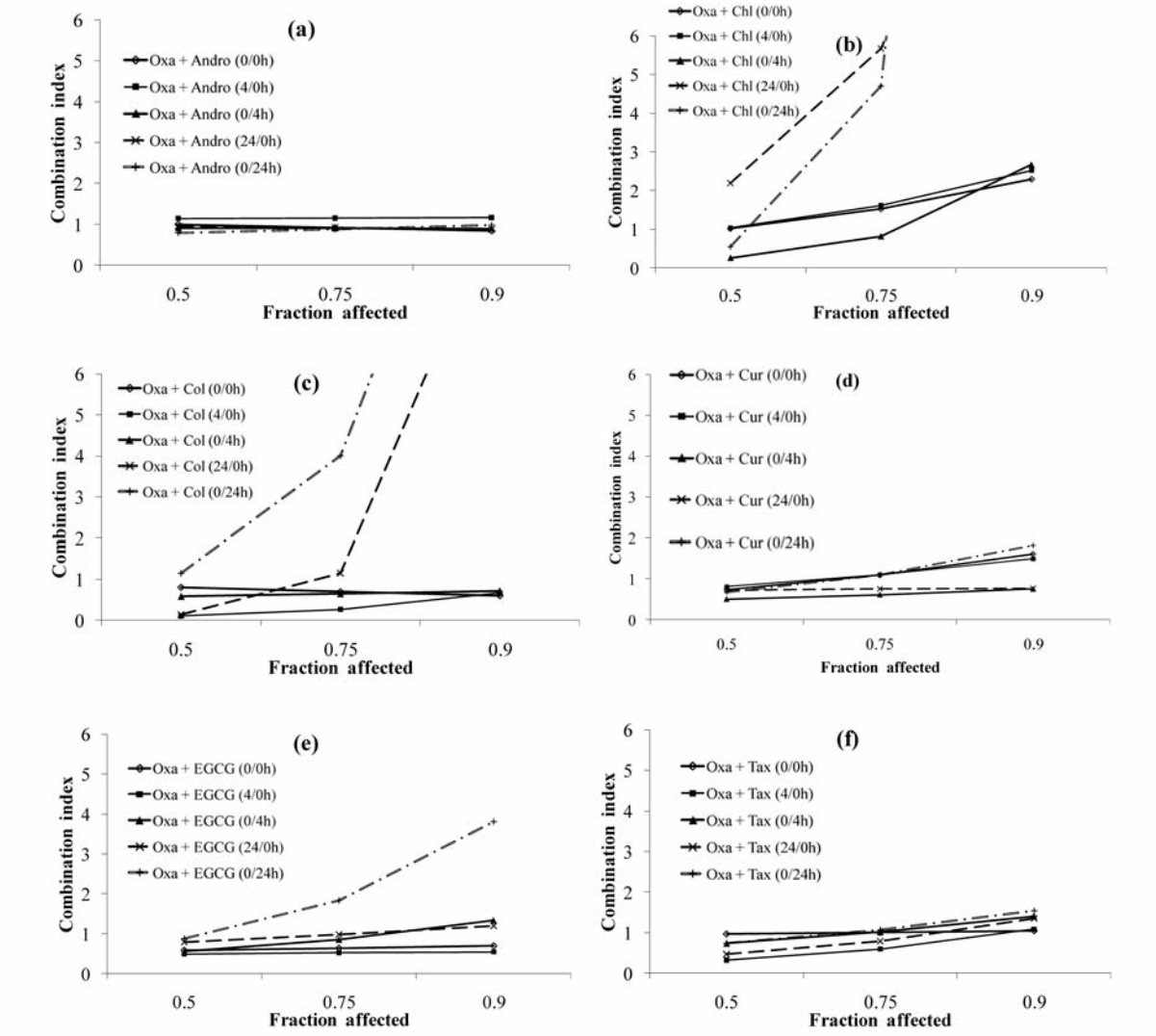

Figure 2. *CIs applying to combination of oxaliplatin with (a) andrographolide, (b) chlorophyllin, (c) colchicine, (d) curcumin, (e) epigallocatechin-3-gallate and (f) paclitaxel using five different sequences of administration of oxaliplatin/phytochemical (when 0h was time of administration of first drug) in A2780^{CisR} cells. ED: Effective dose, % cell kill.*

been reported. EGCG was shown to be cytotoxic in various cancer cell lines including those of the colon (18), bladder, stomach (19), prostate (20) and ovarian (21, 22) cancers. It can be classified as a cell cycle phase-specific anticancer agent killing cancer cells through apoptosis. EGCG has been shown to arrest rat hepatoma and melanoma cells (23). Chl is a commonly used food additive and internal deodorant for geriatric and osteotomy patients. It kills colon and breast cancer cells through apoptosis that are mediated by cytochrome C and caspase 8 (24, 25). Both EGCG and Chl were reported to arrest the cancer cells at the G_1 phase (23, 25). Andro has also been found to have antitumour activity in ovarian, prostate, colon, lung, renal, epidermoid and leukaemia cancer cell lines (26). Cur was found to be cytotoxic against ovarian cancer cell lines causing cancer cell death through apoptosis (27, 28).

Besides antitumour activity, a number of the phytochemicals used in this study (particularly Cur, Chl and EGCG) act as antioxidants (and therefore can protect cells from oxidative damage) whereas Oxa by acting as a pro-oxidant (due to binding with cellular thiols) generate oxidative stress (29). Based on the protective role played by the phytochemicals, it is difficult to explain why the sequence of 0/4 h combination of Oxa with the phytochemicals gave greater synergism. Perhaps the determination of cellular accumulation of platinum, level of platinum-DNA binding and proteomic studies to characterize the key proteins responsible for drug resistance and monitoring of oxidative stress in the cell, may provide further insight.

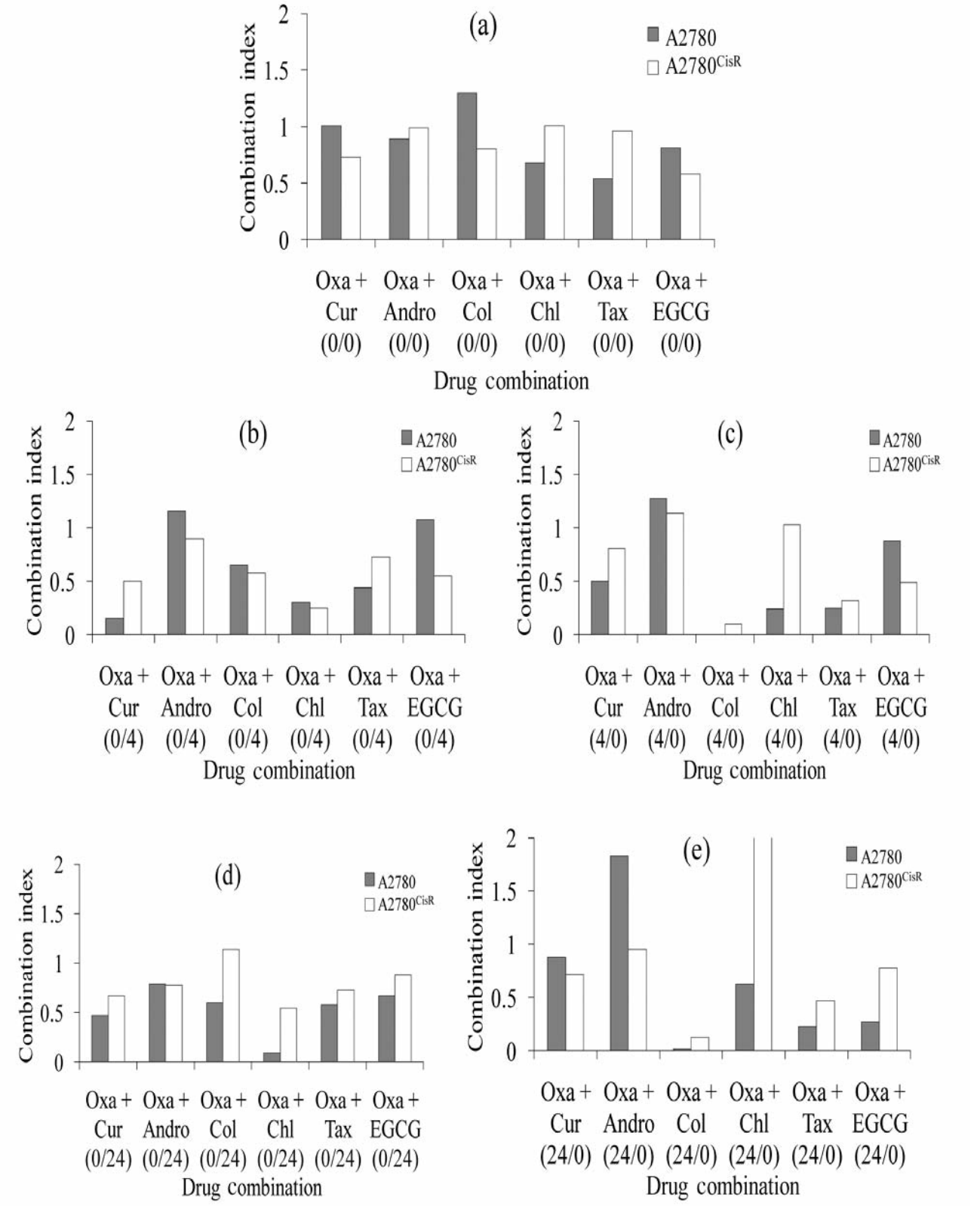

Figure 3. *Combination indices at ED_{50} applying to combinations of oxaliplatin with andrographolide, chlorophyllin, colchicine, curcumin, epigallocatechin-3-gallate and paclitaxel using five different sequences of administration (when 0h was time of administration of first drug) in A2780 and A2780^{cisR} cells.*

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