Anti-proliferative and Pro-apoptotic Effects from Sequenced Combinations of Andrographolide and Cisplatin on Ovarian Cancer Cell Lines

NURHANAN M. YUNOS[1], SITI S.M. MUTALIP[1], MUHAMMAD H. JAURI[1], JUN Q. YU[2] and FAZLUL HUQ[2]

[1]*Drug Discovery Centre, Natural Products Division, Forest Research Institute Malaysia, Kepong, Selangor, Malaysia;*
[2]*Discipline of Biomedical Science, Sydney Medical School, University of Sydney, Lidcombe, Australia*

Abstract. *Andrographolide (Andro) is a diterpenoid that is isolated from Andrographis paniculata and reported to be active against several cancer cell lines. However, few in-depth studies have been carried out on its effects on ovarian cancer cell lines alone or in combination with cisplatin (Cis), which is commonly used to treat ovarian cancer. The aim of this study was to determine the anti-proliferative and apoptotic effects of Andro administered alone and in combination with Cis in the ovarian A2780 and A2780^{cisR} cancer cell lines using five different sequences of administration (Cis/Andro h): 0/0h, 4/0 h, 0/4 h, 24/0 h and 0/24 h. The results were evaluated in terms of medium-effect dose (D_m) and combination indices (CI) using the CalcuSyn software. Unlike Cis, whose activity was lower in the resistant A2780^{cisR} cell line than in the parent A2780 cell line, Andro was found to be three times more active in the A2780^{cisR} cell line as compared to that in A2780 cell line. Synergism was observed when Cis and Andro were administered using the sequences 0/4 h and 4/0 h. The percentage of apoptotic cell death was found to be greater for the 0/4 h combination of Andro and Cis as compared to those values from single-drug treatments. The results may be clinically significant if confirmed in vivo.*

In terms of mortality rates, ovarian cancer ranks high among all types of gynaecological cancers, often detected at an advanced stage due to late diagnosis. Cisplatin, the first platinum drug to enter the clinic continues to be widely used to treat various types of cancer, including advanced ovarian cancer. However, the problems of drug resistance (intrinsic and acquired) and the toxic side-effects (*e.g.* ototoxicity, nephrotoxicity, and neurotoxicity) continue to be main factors limiting the efficacy of cisplatin and other platinum drugs. Hence the search for better anticancer agents for ovarian cancer continues.

One of the possible ways to increase the efficacy of cisplatin in terms of overcoming drug resistance and reducing the side-effects is to combine it with potent bioactive compounds having different modes of antitumour action (1). Whereas platinum resistance is associated with anti-apoptotic factors and pathways such as (NF-κB) and (AKT), a number of phytochemicals have been shown to reduce expression of such proteins so that tumour-active phytochemicals may be ideal candidates for such combination. In this study, the phytochemical andrographolide, which is a bioactive diterpenoid isolated from *Andrographis paniculata*, was combined with cisplatin with the aim of providing a means of overcoming drug resistance in ovarian cancer. It may be noted that *A. paniculata* has several medicinal properties including anti-hypertensive, anti-inflammatory, anti-malarial actions, and is consumed as a herbal ingredient and extract (2-4). Andrographolide has been reported to affect multiple proteins targets (5, 6), whereas cisplatin is known to cause DNA damage (7), which initiates multiple events in the cell cycle which are responsible for programmed cell death. The aim of this study was to determine the anti-proliferative effect of andrographolide administered alone and in combination with cisplatin in two ovarian cancer cell lines, the cisplatin-sensitive A2780 and -resistant A2780^{cisR} cell lines.

Materials and Methods

Materials. Cisplatin was prepared based on the Dhara method (8). Andrographolide was purchased from Sigma-Aldrich, Sydney, Australia. Foetal calf serum (FCS), RPMI 1640, 200 mM L-glutamine, and 5.6% sodium bicarbonate were obtained from Trace

Correspondence to: Fazlul Huq, Discipline of Biomedical Science, The University of Sydney, 75 East Street, Lidcombe NSW 1825, Australia. Tel: +61 293519522, Fax: +61 293519520, e-mail: Fazlul.huq@sydney.edu.au and Nurhanan Murni Yunos, Drug Discovery Centre, Natural Products Division, Forest Research Institute Malaysia, Kepong, Selangor, Malaysia. E-mail: hanan@frim.gov.my

Key Words: Andrographolide, cisplatin, ovarian cancer, apoptosis, synergism, anti-proliferative.

Biosciences Pty Ltd, Sydney, Australia. Other chemicals were purchased from Sigma, Sydney Australia unless stated otherwise. The ovarian cancer cell lines were generous gifts from Ms. Mei Zhang, Royal Prince Alfred Hospital, Sydney, Australia. Stock solutions of platinum compounds (1 mM) were prepared in (DMF-mQ) water mixture and those of plant compounds (1 mM) were prepared in ethanol.

Cell culture. Human ovarian cancer cell lines A2780 (cisplatin-sensitive, parental cell line), and A2780^{cisR} (cisplatin-resistant) were seeded in 25-cm^2 tissue culture flasks in carbon dioxide incubator at 37˚C in a humidified atmosphere consisting of 5% CO_2 in air. The cells were maintained in logarithmic growth phase in a complete medium consisting of RPMI 1640, 10% heat-inactivated FCS, 20 mM Hepes, 0.112% bicarbonate, and 2 mM glutamine (9). Each cell line was seeded at a density of 4,000 to 5,500 cells/well in the 96-well plates in 10% FCS/RPMI 1640 culture medium and left overnight in the carbon dioxide incubator for treatment.

Single-drug treatments. For single-drug treatments, stock solutions were subjected to serial dilutions to give final concentrations ranging from 0.005 to 10 μM. The dilutions were made using 10% RMPI 1640 medium without serum as the vehicle added to equal volumes of cell culture in triplicate wells, then cells were left to incubate in the carbon dioxide incubator for 72 h. Treatments were carried out to determine (IC_{50}) values.

Combination studies. In combination studies, cells were treated with solutions of compounds alone and in combination at three different concentrations, generally at constant ratios of their IC_{50} values. One hundred microliters of cells were seeded in each well of a 96-well plate prior to treatment with single and combinations of compounds. Cisplatin was combined with andrographolide according to the following five sequences of administration: a) simultaneous addition (0/0 h), b) Cisplatin administered first followed by Andro 4 h later (0/4 h), c) andrographolide administered first followed by cisplatin 4 h later (4/0 h), d) cisplatin administered first followed by andrographolide 24 h later (0/24h) and e) andrographolide administered first followed by cisplatin 24 h later (24/0 h). Non-treated cells served as control. The plates containing cells and compounds were left to incubate under normal growth conditions for a period of 72 h from the time of addition of the first compound. The inhibition of the cell growth was determined using the (MTT) reduction assay (10). At the end of 72 h of incubation, MTT (50 μl per well of 1 mg/ml MTT solution) was added to each plate. Four hours after the addition of MTT, the yellow formazan crystals produced from the reduction of MTT was dissolved in 150 μl of DMSO and the absorbance of the resulting solution was read using Bio-Rad Model 3550 microplate Reader (Agilent) set at 570 nm. The absorbance values of the treated and untreated (control) cells were used to calculate growth inhibition due to single-drug treatment and that due to drug combinations. In the case of treatment with single drugs, IC_{50} values (*i.e.* concentrations of compounds needed to cause 50% cell kill) were obtained from the dose response curves whereas in the case of drug combinations, combination index (CI) values were obtained to provide a quantitative measure for combined drug action in terms of synergism, additiveness or antagonism. Each experiment was repeated at least three times.

Table I. *Inhibitory concentration (IC_{50}) values (μM) and resistance factors (RF) for andrographolide and cisplatin, as applied to the human ovarian cancer cell lines A2780 and A2780^{cisR}.*

Drug	A2780	A2780^{cisR}	RF^c
Andrographolide	19.69±1.10	6.66±1.66	0.34
Cisplatin	1.45±0.52	6.64±0.14	4.58

Combination analysis. CIs used as measures of combined drug action were calculated using the program CalcuSyn (11, 12). The (CI) for binary combination of drugs is defined by the following equation:

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

D_1 and D_2 represent the mean doses of compounds 1 and 2 respectively, in combination required to cause x% inhibition, whereas D_{1x} and D_{2x} represent the doses of compounds 1 and 2 required to cause x% inhibition when present alone. D_x can be readily calculated from the following form of the median-effect equation:

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

where 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.

CI values of <1, =1 and >1 indicate synergism, additivity and antagonism in combined drug action, respectively.

DNA fragmentation assay (apoptosis assay). DNA fragmentation, as a measure of apoptotic cell death following drug treatment, was assayed using Deadend™ Colorimetric Apoptosis Detection System (Promega, Madison, USA). In brief, 1×10^5 cells were plated into four sets of chamber slides and incubated in an atmosphere of 5% CO_2 for 24 h. Following this, cells were treated with andrographolide and cisplatin alone on separate slides. For studies on combination between cisplatin and andrographolide, cisplatin was administered first followed by andrographolide 4 h later. Cells treated with DNase 1 served as the positive control. The apoptosis assay was carried out according to the manufacturer's protocol. The apoptotic cells (that produced yellow fluorescence) and non-apoptotic cells (that produced red colour) were examined under a fluorescence microscope (13). The experiments were repeated at least three times. The percentage of apoptotic cells was determined as follows.

$$\% \text{ of apoptotic cells} = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells}} \times 100$$

All experiments were performed at least in triplicate and the data are presented as the mean±S.D.

Table II. *Dose-effect parameters (D_m), (m) and (r) for the combinations of cisplatin and Andrographolide in A2780 and A2780^{cisR} cell lines.*

Sequence of administration (cisplatin/andrographolide)	A2780			A2780^{cisR}		
	D_m	m	r	D_m	m	r
0/0 h	1.52	0.62	1.00	3.27	1.20	1.00
4/0 h	2.81	0.91	1.00	2.49	0.89	0.97
0/4 h	1.83	0.81	1.00	1.52	0.83	0.98
24/0 h	2.32	0.69	0.99	3.79	1.17	0.99
0/24 h	1.19	0.57	1.00	1.64	0.86	0.98

Results

Anti-proliferative activities of andrographolide and cisplatin. The median effect doses (D_m also denoted as ED_{50}) of andrographolide and cisplatin applying to A2780 and A2780^{cisR} cell lines are summarised in Table I. The resistance factor (RF) is defined as the ratio of the drug concentration required for 50% cell kill in the resistant cell line to that in the parental cell line. Cisplatin was less active in the resistant cell lines than in the parental cell line. The converse was true for Andro although Cis was more active than andrographolide against both cell lines.

The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments, and are given as the means±standard deviation (SD).

The results are averages of those obtained from four identical wells with 5,000 cells per well. RF: Ratio of IC_{50} value for A2780^{cisR} to A2780 cell line.

Anti-proliferative activities from sequenced combinations between cisplatin and andrographolide. Table II gives dose-effect parameters in terms of median-effect dose, shape (sigmoidicity), conformity (linear correlation coefficient), represented as Dm, m and r respectively as applied to the combinations of cisplatin and andrographolide in A2780 and A2780^{cisR} cell lines. Figure 1 gives the CIs applying to sequenced combinations of cisplatin and andrographolide at ED_{25}, ED_{50}, ED_{75} and ED_{90} (meaning drug concentrations required for 25%, 50%, 75% and 90% cell kill, respectively) in A2780 and A2780^{cisR} cell lines, for which the CIs were also denoted respectively as CI_{50}, CI_{75} and CI_{90}.

From Figure 1, it can be seen that lower concentrations for combinations of cisplatin and andrographolide were mostly antagonistic, irrespective of sequence of administration. At higher concentrations, synergism in cell kill was observed when the two compounds were administered with a time gap. In contrast, the bolus administration was antagonistic-to-slightly synergistic in the parental A2780 cell line and antagonistic-to-additive in the resistant A2780^{cisR} cell line. A more careful analysis of the results from administration with a time gap shows that somewhat greater synergism was produced when cisplatin was administered first followed by andrographolide 4 or 24 h later (0/4 h and 0/24 h sequences). For the 0/4 h sequence of administration, generally a greater synergism was seen in the A2780^{cisR} cell line (CI_{25}=0.52; CI_{50}=0.66; CI_{75}=0.84) than in the parental A2780 cell line (CI_{25}=3.65; CI_{50}=1.51; CI_{75}=0.66) especially at lower drug concentrations. The results are different from those observed for the combination of cisplatin and oxaliplatin with quercetin and thymoquinone in A2780 and A2780$_{cisR}$ cell lines, where a greater synergism was observed when the phytochemical was administered first, followed by the platinum drug 2 h later (14). These are further discussed in this article.

DNA damage as a hallmark of apoptosis. DNA damage, being one of the hallmarks of apoptosis, and DNA fragmentation in A2780 and A2780^{cisR} cells due to treatments with cisplatin and andrographolide and their combinations, were assessed by using Deadend™ Colorimetric Apoptosis the Detection System. As shown in Figure 2, cells treated with DNAse served as a positive control. Yellow fluorescence indicates the occurrence of DNA fragmentation. The non-treated cells did not exhibit yellow fluorescence, indicating the absence of apoptosis. Greater cell death and apoptosis were observed in cells treated with cisplatin and its combination with andrographolide. Figure 2 gives a visual picture of apoptotic cells in A2780 and A2780^{cisR} cells when they were treated with andrographolide, cisplatin and their combination using 0/4 h sequence of administration, as observed under fluorescence microscopy (magnification: ×200). Figure 3 gives the corresponding percentages of apoptotic cells in A2780 and A2780^{cisR} cell lines when they were treated with andrographolide and cisplatin and their combination (using 0/4 h sequence of administration).

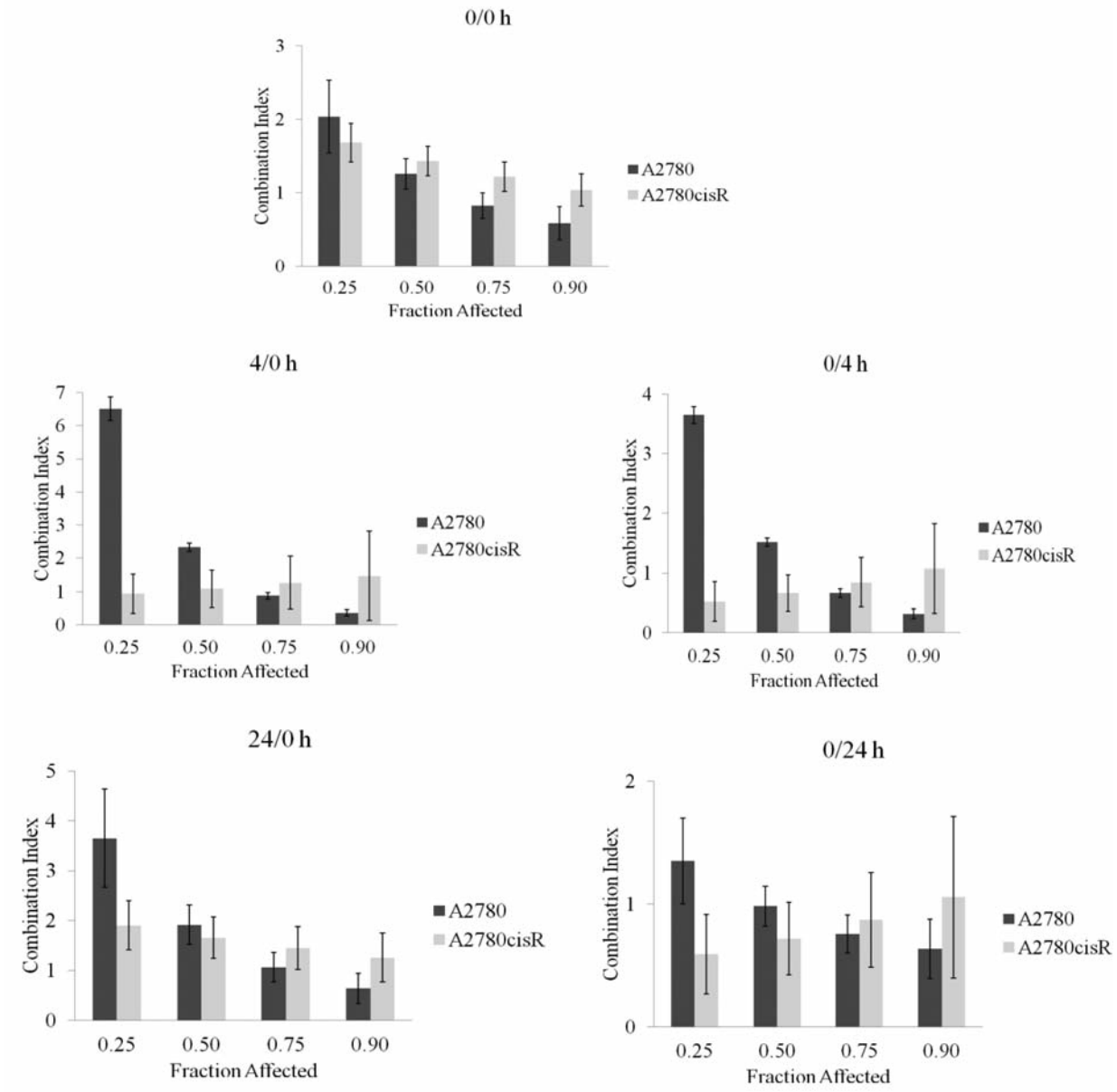

Figure 1. *Combination indices (CIs) applying to combinations of andrographolide and cisplatin administered using 0/0 h, 4/0 h, 0/4 h, 24/0 h and 0/24 h sequences in A2780 and A2780^{cisR} cell lines.*

Discussion

Although cisplatin was found to be more active than Andro towards both the parental A2780 and the resistant A2780^{cisR} cell lines, andrographolide had a higher activity in the resistant cell lines than in the parental cells. In contrast, activity of cisplatin was found to be lower in the resistant cell lines. The results indicate that andrographolide was able to overcome mechanisms of resistance operating in A2780^{cisR} and A2780^{ZD0473R} cell lines. When sequenced combinations of andrographolide and cisplatin were applied to human ovarian A2780 and A2780^{cisR} cancer cell lines, strong synergism (CI<1) was observed when the compounds were administered using a 4-h time gap (as in 0/4 h and 4/0 h sequences) in the resistant A2780^{cisR} cell line especially at higher concentrations. A more careful analysis shows that somewhat greater synergism was produced when cisplatin was administered first followed by andrographolide 4 or 24 h later (0/4 h and 0/24 h sequences). As noted earlier, the results are different from those observed with the

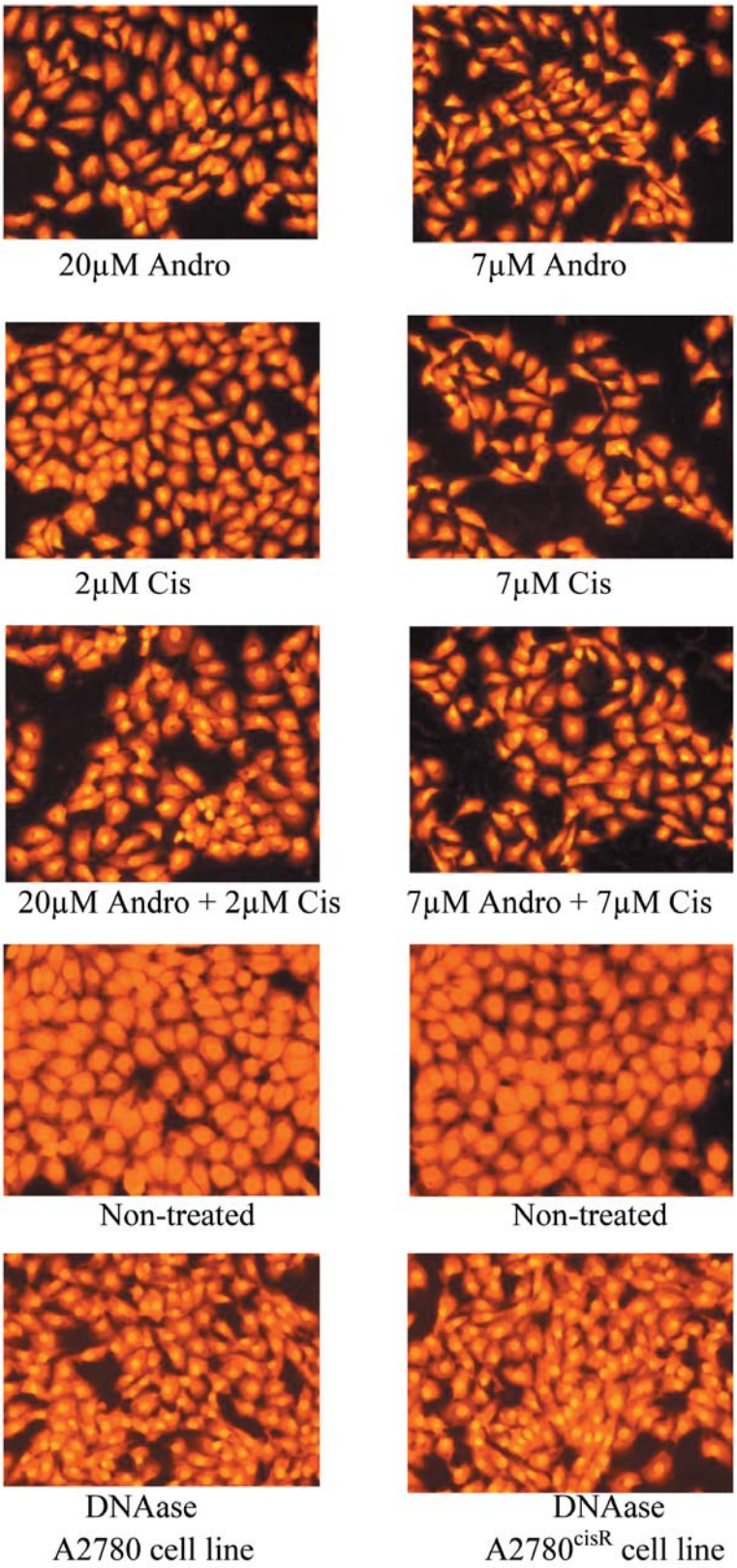

Figure 2. *Visualisation of apoptotic cells in A2780 and A2780^{cisR} under the fluorescence microscope when the cells were treated with andrographolide (Andro), cisplatin(Cis) and 0/4 h combination of the two. Magnification: ×200.*

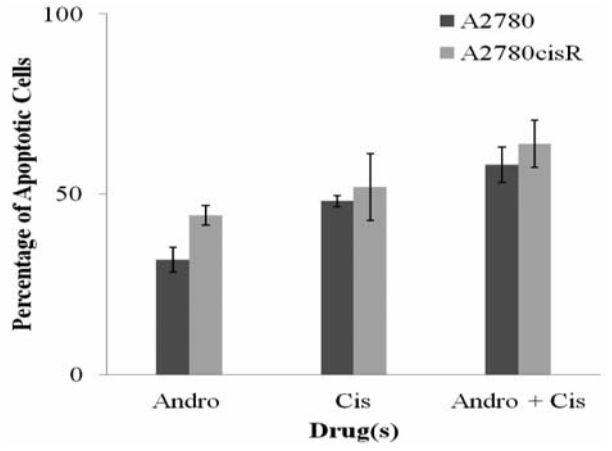

Figure 3. *Percentage of cells undergoing apoptotic cell death in A2780 and A2780^{cisR} cell lines when they were interacted with andrographolide (Andro) and cisplatin (Cis) and their combination using 0/4 h sequence.*

combination of cisplatin and oxaliplatin with quercetin and thymoquinone in A2780 and A2780^{cisR} cell lines, where a greater synergism was observed when the phytochemical was administered first, followed by the platinum drug 2 h later (14). On a qualitative level, quercetin, thymoquinone and andrographolide have common functions, namely as anti-oxidants and as cytotoxic agents in inducing cell kill. However, these compounds may differ in the exact mechanisms of anticancer action. It has been found that andrographolide inhibits the activation of p38 (MAPKs) but not that of (JNK), (ERK1/2) and NF-κB (15). In contrast, anticancer action of quercetin and thymoquinone is found to be associated with inhibition of transcription factor NF-κB and AKT pathways. It has been suggested that the anti-inflammatory effect of andrographolide may be resulting from the inhibition of (iNOS) and (COX-2) expression through inhibition of p38 MAPK activation (16).

It has recently been reported that andrographolide is capable of sensitizing cancer cisplatin-induced cell death (17). Andrographolide has also been reported to sensitize the apoptotic action of 5-fluorouracil in hepatocellular carcinoma SMMC-7721 cells *via* caspase-8–dependent mitochondrial p53 pathway (18) and that of gemicitabine in pancreatic cancer (19).

As to the question why generally the 0/4 h sequence of administration (*i.e.* cisplatin followed by andrographolide after four hours) was found to be more synergistic than the converse and the bolus, it may be noted that andrographolide is a cell cyle phase-specific drug that was reported to cause cell cycle arrest at the G_1/S phase in hepatoma, colorectal and leukaemia cells (6, 19-20). This is believed to be due to increased expression of cell cycle-inhibitory proteins, including p16, p21 and p27, that are associated with the reduced expression of cyclins required for G- to S-phase transition (19). However, whether andrographolide has a similar cell-cycle effect on ovarian cancer cells or not is yet to be confirmed. The key players in apoptosis due to andrographolide are caspases (in extrinsic death receptor pathway), (BCL-2) family members including (BID) and (BAX); involved in intrinsic apoptosis pathway) and (TRAIL; also an important member in extrinsic apoptosis pathway) (21). Andrographolide was also reported to act at the G_2/M phase in human glioblastoma cells and to decrease the signaling of PI3K/AKT (22). In contrast, cisplatin is not considered as being a cell cycle phase-specific drug, although it is known to be sensitive at G_1 phase in the cell cycle (1). Chemoresistance due to cisplatin in ovarian cancer cells is associated with inactivation of AKT signaling protein (PTEN). PTEN is a tumour suppressor protein that acts as a central negative regulator of the PI3K/AKT signaling cascade and suppresses both cell survival and cell proliferation (23). Decrease in expression of PTEN can lead to increased phosphorylation and activation of AKT, further promoting cell survival and proliferation. It remains to be seen whether any of these mechanisms apply to the tested ovarian cancer cell lines.

The percentages of apoptotic cells was higher when the cells were treated with combinations of cisplatin and andrographolide than the single treatments. This is not unexpected when we note that cisplatin and andrographolide bring about apoptosis by distinctly different mechanisms (20, 23-25).

In conclusion, the results of the present study indicate that combined drug action from sequenced combinations of cisplatin and andrographolide varies from synergistic to antagonistic, dependent on the sequence of administration and concentration used. The results can be seen to support the idea that appropriate sequenced combination of targeted therapy and phytochemical can provide a means of overcoming cancer cell drug resistance.

Conflicts of Interest

Nurhanan Murni Yunos, Siti Syarifah, Muhammad Haffiz, Jun Qing Yu and Fazlul Huq declare that they have no financial and personal relationships with other people or organizations that could inappropriately influence their work.

Acknowledgements

Nurhanan Murni Yunos is grateful to the Government of Malaysia for the award of a full Ph.D. scholarship.

References

1 Shah MA and Schwartz GK: The relevance of drug sequence in combination chemotherapy. Drug Resist Update *3*: 335-336, 2000.

2 Sharma A, Lal K and Handa SS: Standardization of the indian crude drug kalmegh by high-pressure liquid-chromatographic determination of andrographolide. Phytochem Anal *3(3)*: 129-131, 1992.
3 Nanduri S, Nyavanandi VK, Thunuguntla SSR, Kasu S, Pallerla MK, Ram PS, Rajagopal S, Kumar RA, Ramanujam R, Babu JM, Vyas K, Devi AS, Reddy GO and Akella V: Synthesis and structure-activity relationships of andrographolide analogues as novel cytotoxic agents. Bioorg Medicinal Chem Lett *14(18)*: 4711-4717, 2004.
4 Huq F and Yunos NM: Molecular modelling analysis of the metabolism of andrographolide. IJPAC *2*: 359-369, 2007.
5 Kumar RA, Sridevi K, Kumar NV, Nanduri S and Rajagopal S: Anticancer and immunostimulatory compounds from *Andrographis paniculata*: J Ethnopharmacol *92(2-3)*: 291-295, 2004.
6 Shen KK, Liu TY, Xu C, Ji LL and Wang ZT: Andrographolide inhibits hepatoma cells growth and affects the expression of cell cycle related proteins. Yao Xue Xue Bao *44(9)*: 973-979.
7 Lippard SJ: Chemistry and molecular biology of platinum anticancer drugs. Pure Appl Chem *59(6)*: 731-742, 1987.
8 Dhara SC: A rapid method for the synthesis of cis-$[Pt(NH_3)_2Cl_2]$. Indian J Chem *8*: 193-94, 1970.
9 Yunos NM, Beale P, Yu JQ and Huq F: Synergism from the sequence combinations of curcumin and epigallocatechin-3-gallate with cisplatin in the killing of human ovarian cancer cells. Anticancer Res *31*: 1131-1140, 2011.
10 Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods *65(1-2)*: 55-63, 1983.
11 Chou TC and Talalay P: Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*: 27-55, 1984.
12 Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev *58(3)*: 621-812, 2006.
13 Al Batran R, Al-Bayaty F, Al-Obaidi MM and Abdulla MA: Acute Toxicity and the effect of andrographolide on porphyromonas gingivalis-induced hyperlipidemia in rats. Biomed Res Int *2013*: 594012 , 2013.
14 Nessa MN, Beale P, Chan C, Yu JQ and Huq F: Synergism from combinations of cisplatin and oxaliplatin with quercetin and thymoquinone in human ovarian tumour models, Anticancer Res *31*: 3789-3798, 2011.
15 Ghosh N, Ghosh R, Mandal V and Mandal SC: Recent advances in herbal medicine for treatment of liver diseases. Pharmaceut Biol *49(9)*: 970-988, 2011.
16 Liu J, Wang ZT, Ji Li and Ge BX: Inhibitory effects of neoandrographolide on nitric oxide and prostaglandin E2 production in LPS-stimulated murine macrophage, Mol Cell Biochem *298*: 49-57, 2007.
17 Zhou J, Hu Se, Tan SH, Cao R, Chen Y, Xia D, Zhu X, Yang XF, Ong CN and Shen HM: Andrographolide sensitizes cisplatin-induced apoptosis *via* suppression of autophagosome-lysome fusion in human cancer cells. Autophagy *8(3)*: 338-349, 2012.
18 Yang L, Wu D, Luo K, Wu S and Wu P: Andrographolide enhances 5-fluorouracil-induced apoptosis *via* caspase-8-dependent mitochondrial pathway involving p53 participation in hepatocellular carcinoma (SMMC-7721) cells. Cancer Lett *276(2)*: 180-188, 2009.
19 Shi MD, Lin HH, Lee YC, Chao JK, Lin RA and Chen JH: Inhibition of cell-cycle progression in human colorectal carcinoma Lovo cells by andrographolide. Chem Biol Interact *174(3)*: 201-210, 2008.
20 Cheung HY, Cheung SH, Li J, Cheung CS, Lai WP, Fong WF and Leung FM: Andrographolide isolated from *Andrographis paniculata* induces cell cycle arrest and mitochondrial-mediated apoptosis in human leukemic HL-60 cells. Planta Medic *71(12)*: 1106-1111.
21 Zhou J, Zhang S, Ong CN and Shen HM Critical role of pro-apoptotic Bcl-2 family members in andrographolide induced apoptosis in human cancer cells. Biochem Pharmacol *72*: 132-144, 2006.
22 Li Y, Zhang P, Qiu F, Chen L, Miao C, Li J, Xiao W and Ma E: Inactivation of PI3K/Akt signaling mediates proliferation inhibition and G_2/M phase arrest induced by andrographolide in human glioblastoma cells. Life Sci *90(25-26)*: 962-967.
23 Singh M, Chaudhry P, Fabi F and Asselin E: 2013: Cisplatin-induced caspase activation mediates PTEN cleavage in ovarian cancer cells: A potential mechanism of chemoresistance. BMC Cancer *13*: 233-241, 2013.
24 Tee TT and Azimahtol HL: Induction of apoptosis by Eurycoma longifolia jack extracts. Anticancer Res *25(3B)*: 2205-2213, 2005.
25 Bao GQ, Shen BY, Pan CP, Zhang YJ, Shi MM and Peng CH: Andrographolide causes apoptosis *via* activation of STAT3 and AKT. Toxicol Lett *222(1)*: 23-35, 2013.

Received August 18, 2013
Revised September 18, 2013
Accepted September 19, 2013