# Studies on Combinations of Platinum with Paclitaxel and Colchicine in Ovarian Cancer Cell Lines

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**Abstract.** *Ovarian cancer remains an ongoing challenge because of the occurrence of resistant forms of tumour for which the drugs fail to function. Combination therapy using drugs with different mechanisms of action offer a means of overcoming drug resistance and reducing the side-effects. In this study, binary combinations of four platinum compounds cisplatin (Cs), oxaliplatin (Ox), YH12 [trans-$PtCl_2$(ammino) {imidazo-(1,2-α)pyridine}] and TH1 [{trans-$PtCl(NH_3)_2$}$_2$ {trans-Pt(3-hydroxypyridine)$_2$($H_2N(CH_2)$ $6NH_2$)$_2$}$Cl_4$] and two plant-based mitotic inhibitors paclitaxel (Tx) and colchicine (Co) have been used against ovarian cancer cell lines A2780 and A2780$^{cisR}$ using five different sequences of addition: 0/0 h, 4/0 h, 0/4 h, 24/0 h and 0/24 h. The strongest synergistic effect was observed when the plant compound (Tx or Co) was added first followed by platinum four hours later with combination index at 50% effect level (fa= 0.5) ranging from 0.03 to 0.36 and 0.10 to 0.72 in A2780 and A2780$^{cisR}$ cells respectively. Of all the platinum compounds, Cs showed the greatest synergism when combined with Tx and Co (combination index, $CI_{50}$=0.03 in A2780 and from 0.10 to 0.12 in A2780$^{cisR}$). With the sequence 24/0 h, platinum compounds showed greater synergistic effect with Co than Tx in A2780$^{cisR}$. With the sequences 0/4 h and 0/24 h, most of the combinations showed weak synergism to antagonism, especially in A2780$^{cisR}$. Antagonism was also observed when the two compounds were added simultaneously, especially in A2780$^{cisR}$. Conclusion: Binary combinations of platinum compounds Cs, Ox, YH12 and TH1 with plant compounds Tx and Co applied to ovarian cancer cell lines showed sequence- and concentration-dependent synergism. The results may have profound implications in therapy, if found to be true in vivo.*

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Although the incidence of ovarian cancer is lower than that of breast and colorectal cancer, the fatality rate is much greater (1), making it the leading cause of death from gynaecological cancer in the Western world (2). This may be due to poor prognosis and the absence of early symptoms so that at the time of diagnosis, the disease would have spread beyond the ovaries in approximately two-thirds of patients (3).

Platinum drugs such as cisplatin (Cs) and oxaliplatin (Ox) are routinely used to treat various types of cancer including ovarian cancer. Whereas Cs is highly effective against testicular and ovarian cancer and has proved beneficial also in the treatment of head and neck, lung and bladder cancer (4), Ox is used to treat colorectal cancer (5) and has some beneficial effect against ovarian cancer as well (6). In spite of their widespread use, both Cs and Ox suffer from major drawbacks related to side-effects and resistance (7, 8). Hence intense research effort has been applied to arrive at new platinum compounds with reduced side-effects and a wider spectrum of activity.

Although Cs is tumour active, transplatin is inactive but toxic due to its higher reactivity so that the compound is essentially deactivated before binding with DNA. Following the suggestion that the *trans* geometry in platinum(II) can be activated for antitumour activity by the introduction of bulky ligands, a number of tumour active *trans*-planaramin-platinum(II) complexes have been prepared (9-11). One such compound code named YH12 has one imidazo(1,2-α)pyridine ligand, one $NH_3$ and two $Cl^-$ bound to platinum(II) in a trans geometry. YH12 is significantly more active than Cs against ovarian A2780$^{cisR}$ cancer cell line (11). Multicentred platinums such as BBR3464 and TH1 (which has two 3-hydroxypyridine ligands bound to the central platinum) (12, 13) constitute another class of highly tumour active platinum compounds. Like YH12, TH1 is more active than Cs against the cell line A2780$^{cisR}$. Whereas Cs binds with DNA forming mainly bifunctional intrastrand

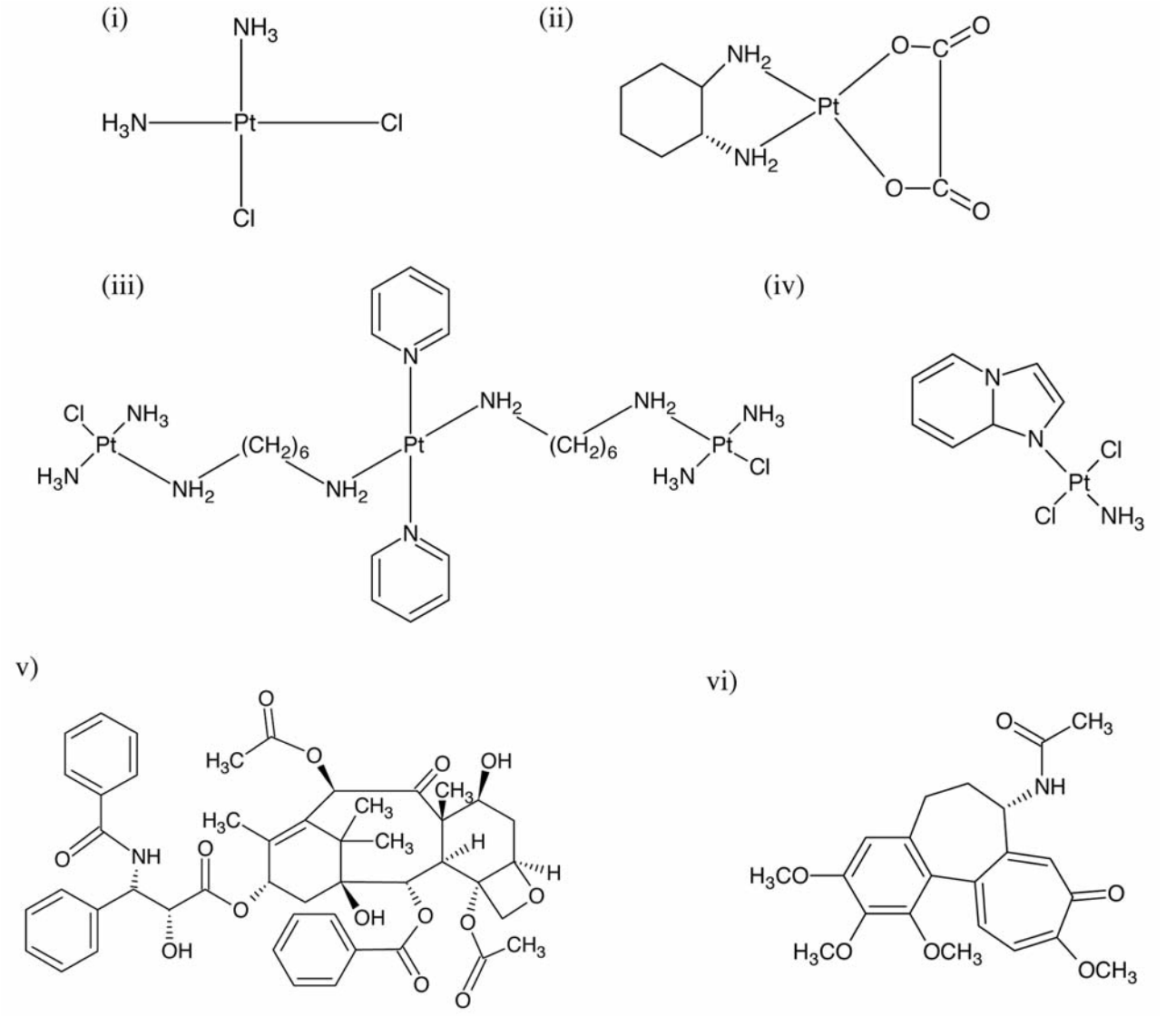


Figure 1. *Chemical structures of i) cisplatin, ii) oxaliplatin, iii) TH1, iv) YH12, v) paclitaxel, vi) colchicine.*

adducts Pt(GG) and Pt(AG), YH12 and TH1 are expected to form mainly interstrand GG adducts.

There is an increasing interest in phytochemicals as they provide a natural source of drugs for the treatment of various diseases, including cancer. Two such tumour active compounds are paclitaxel (Tx) and colchicine (Co) that act as microtubule inhibitors and cause cell cycle arrest at the mitotic phase (14, 15). The chemical structures of Tx and Co, along with those for Cs, YH12, Ox and TH1 are given in Figure 1. Although both Tx and Co cause cell death instead of cell division, Tx promotes polymerisation of tubulin, whereas Co causes depolymerisation (16). Since availability of tubulin is essential for mitosis, both Tx and Co effectively function as mitotic poisons.

Natural therapies are being increasingly used by cancer patients, together with targeted therapies, although little is known about their interaction with conventional chemotherapeautic agents. In this study, synergism in activity from combinations of Cs, YH12, Ox and TH1 with Tx and Co in human ovarian cancer cell lines A2780 and $A2780^{cisR}$ were investigated as a function of both concentration and sequence of addition. Besides providing information on combined drug interaction, the study also aimed to provide a means of overcoming drug resistance and reducing therapy side-effects (17, 18). Currently, Tx and Cs are used in combination as the first line of treatment against ovarian cancer; although the lifespan has been increased by 4 to 5 years as compared to a year from single

treatment, this increased effect is observed for only 50% of patients (19, 20).

## Materials and Methods

*Materials*. Cs, YH12 and TH1 were prepared according to previously described methods (11, 13, 21). Ox, Tx and Co were 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 Biosciences Pty Ltd Australia. Other chemicals were mostly purchased from Sigma. 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.

*Cell culture*. Human ovarian cancer cell lines A2780 and A2780$^{cisR}$ 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 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 (22). Each cell line was seeded at a density of 4000 to 6000 cells/well in 96-well plates in 10% FCS/RPMI 1640 culture medium and left overnight in the incubator for treatments on the following days.

*Single-drug treatment*. For single drug treatment, each of the stock solutions was subjected to serial dilutions to give final concentrations ranging from 0.005 to 10 μM. The dilutions were performed using 10% RMPI-1640 medium without serum as the vehicle and were added to equal volumes of cell culture in triplicate wells, then cells were left to incubate for 72 h. These treatments were carried out to determine ($IC_{50}$) values *i.e*. drug concentrations required for 50% cell kill.

*Combination analysis*. Dose–response curves and CIs were used as measures of synergism calculated using the program CalcuSyn (24, 25). The CI for binary combinations of drugs was calculated according to the equation:

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

where $D_1$ and $D_2$ represent 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 respectively required to cause x% inhibition when present alone. $D_x$ can be readily calculated from the following form of 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 by the dose, fu 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. The CI values of <1, =1 and >1 indicate synergism, additivity and antagonism in combined drug action, respectively.

Table I. *$IC_{50}$ (μM) values of platinum (Cs, Ox, YH12 and TH1) and plant compounds (Tx and Co) for human ovarian cancer cell lines A2780 and A2780$^{cisR}$.*

| Compound | $IC_{50}$ (μM) | | Resistance factor |
|---|---|---|---|
| | A2780 | A2780$^{cisR}$ | |
| Cs | 1.45 | 6.64 | 4.58 |
| YH12 | 8.46 | 5.30 | 0.63 |
| TH1 | 1.07 | 2.90 | 2.71 |
| Ox | 0.55 | 0.97 | 1.75 |
| Tx | 0.003 | 0.006 | 1.71 |
| Co | 0.02 | 0.02 | 0.98 |

Cs: Cisplatin, Ox: Oxaliplatin, Tx: Paclitaxel, Co: Colchicine.

*Pt cellular accumulation and Pt-DNA binding studies*. As the action of platinum-containing drugs is associated with their binding with DNA, cellular accumulation of platinum and platinum-DNA binding levels in A2780 and A2780$^{cisR}$ cell lines were determined for combinations Cs-Co and Cs-Tx for the sequences 4/0 h and 24/0 h. Whereas the sequence 4/0 h was found to produce pronounced synergism, the sequence 24/0 h produced moderate synergism to antagonism. Combinations of Cs with Co and Tx (at $IC_{50}$ values) were added to culture plates containing exponentially growing A2780 and A2780$^{cisR}$ cells in 10 ml of 10% FCS/RPMI-1640 culture medium (cell density=$1\times10^6$ cells/ml) according to the sequences 4/0 h and 24/0 h. The cells containing the drugs were incubated for at least 48 h counted from the addition of the first compound. The cell monolayers were trypsinized and the cell suspension (10 ml) was centrifuged at 3500 rpm for 2 min at 4˚C. The cells were washed twice with ice-cold phosphate-buffered saline and the pellets were stored at –20˚C until assayed.

*Cellular accumulation*. Cell pellets from drug combinations were suspended in 0.5 ml of 1% Triton-X, held on ice while being sonicated. Total intracellular platinum contents were determined by graphite furnace atomic absorption spectrometry (AAS).

*Platinum-DNA binding*. DNA isolated from the cell pellet using H440050 JETQUICK Blood DNA Spin Kit/50 Austral Scientific Pty Ltd and the modified protocol of Bowtell (26) was determined by UV spectrophotometery at 260 nm and platinum levels were determined by graphite furnace AAS. $A_{260}/A_{280}$ nm ratios were found to be between 1.75 and 1.8 for all samples.

## Results

*Growth-inhibitory effects of single drug*. Table I gives the $IC_{50}$ values of the compounds for the cell lines A2780 and A2780$^{cisR}$. The most active compound is Tx followed by Co. YH12 was the least active compound against the cell line A2780 but it had a higher activity than Cs in the resistant cell line A2780$^{cisR}$.

*Growth-inhibitory effects of drug combinations*. As stated earlier, the main aim of the present study was to investigate

synergism in activity from the combinations of selected platinum compounds and phytochemicals in two human ovarian cancer cell lines. To this end activities of the compounds alone and in combination were determined. Based on the data collected 16 dose- response curves were generated. Tables II and III give dose effect parameters in terms of median-effect dose, shape (sigmoidicity), conformity (linear correlation coefficient) and a new measure of synergism termed enhancement factor (defined more fully later) represented as $D_m$, m, r and EF respectively, as applied to the cell lines A2780 and A2780$^{cisR}$ respectively. Figures 2 and 3 give the dose–response curves for the most synergistic combinations. In all of the combinations given in Figures 2 and 3, the combined drug action is greater than that due to either of the compounds acting alone and the additive sum of the combined action. However, the synergism in activity may not be apparent from the dose-response curves and the Dm values when the compounds differ greatly in their activity. It will be seen later that the CI provides a more clear measure although CI is inversely related to synergism.

A new term called 'enhancement factor' (EF) is defined to give a direct relationship. The enhancement factor at the median effect level ($EF_m$) is defined by the equation:

$$EF_m = \frac{1}{(X_1 \times D_m)/IC50_1 + (X2 \times D_m)/IC50_2}$$

where $X_1$ and $X_2$ are the mole-fractions of drugs 1 and 2 in the mixture, $D_m$ is the total concentration of the drugs required to cause the median effect, $IC50_1$ and $IC50_2$ are the concentrations of drugs 1 and 2 respectively required for 50% cell kill when acting alone.

The following example illustrates the calculation of EF. For the 4/0 h combination of Cs ($IC_{50}$ 1.45 μM) and Co ($IC_{50}$ 0.01 μM) in A2780 cell line at molar ratio 500:1, the concentration of Cs in the mixture equals 0.0398 μM [=(500/501)×0.04 μM] and that of Co equals $7.984\times10^{-5}$ μM [=(1/501)×0.04]. When normalized as multiples of $IC_{50}$, the values (as multiple of $IC_{50}$) are equivalent to (0.0398/1.45=) 0.0275 and ($7.984\times10^{-3}$/0.01=) $7.984\times10^{-3}$ μM respectively so that the normalized $D_m$ as a ratio equals 0.0355, meaning that the two compounds together are able to achieve the same effect (50% cell kill) at a much lower concentration. Put another way, activity has been increased by a factor of 28 (1/0.0355).

Analysis of the EF values indicates that generally the 4/0 h combination of drugs was found to be most synergistic, whereas 0/0 h, 0/4 h and 0/24 h additions were least synergistic, or often antagonistic. Among the platinum drugs, Cs and Ox were found to produce more synergistic combinations (with Tx and Co) than TH1 and YH12. For example, for the 4/0 h combination of Cs with Tx and Co, the $EF_m$ values were 28.0 and 31.5, respectively, whereas the corresponding values with YH12 were 4.0 and 4.2.

*CI of combined drugs: between platinum compounds and Tx or Co*. As stated earlier, besides the use of dose–response curves, the combined drug action was also analyzed using CIs. Table IV gives the dose–effect parameters applying to combinations of platinum compounds with Co and Tx. Table V gives the CI values at median effect level ($f_a$=0.5) applying to the combination of platinums (Cs, YH12, Ox and TH1) with the two phytochemicals (Tx and Co) for different modes of addition namely in bolus and in sequence with 4 h and 24 h gaps. It can be seen that in the A2780 cell line, the combined action of Tx with each of the platinum compounds (Cs, YH12, Ox and TH1) at median effect level is synergistic for all modes of addition except 0/24 h, for which the combination of Tx with TH1 was found to be slightly antagonistic in action. It should be noted that the 4/0 h addition of Tx with platinum compounds was considered to be most synergistic based on dose-response curves and EFs.

As applied to the resistant cell line A2780$^{cisR}$, Tx produced synergistic outcomes with Cs and Ox for all modes of addition except 0/0 h, where the combined action was close to being additive. As applied to the rest of the platinum compounds, the combined action with Tx was synergistic for the addition sequences 4/0 h and 24/0 h whereas for the sequences 0/0 h, 0/4 h and 0/24 h, the combined action was mostly antagonistic. When synergism was shown, generally combinations of Tx with Cs and Ox displayed higher synergism than those with YH12 and TH1. The other phytochemical Co produced high synergism in the A2780 cell line with all the platinum compounds for the addition sequences 4/0 h, 0/4 h, 24/0 h and 0/24 h. As applied to the addition sequence 0/0 h, the combined action of Co with platinum compounds was mostly antagonistic except for Cs where it was synergistic.

The CI values indicate that as applied to the addition sequence 0/0 h, the combined action of Co with platinum compounds in the A2780 cell line was mostly antagonistic and for the addition sequences 0/4 h and 0/24 h, the combined action was synergistic but less so than that for 4/0 h and 24/0 h additions. A similar conclusion was made based on dose–response curves and EF values. In the resistant cell line A2780$^{cisR}$, Co also produced pronounced synergistic outcomes for the addition sequences 4/0 h and 24/0 h, whereas for the sequences 0/0 h and 0/24 h, the combined action of Co with platinum compounds in the A2780$^{cisR}$ cell line was generally antagonistic or close to being additive. As applied to the sequence 0/4 h, the combined action of Co with platinum compounds in the A2780$^{cisR}$ cell line was synergistic but less so than that for sequences 4/0 h and 24/0 h.

Table II. *Dose-effect parameters for the combination between platinum compounds and Tx or Co in A2780 cell line.*

When used alone

| Drug conc. (μM) | Effect | Drug conc. (μM) | Effect |
|---|---|---|---|
| Cs | | Tax | |
| 0.5 | 0.38±0.04 | 0.0005 | 0.18±0.04 |
| 5 | 0.64±0.03 | 0.005 | 0.57±0.02 |
| 50 | 0.84±0.02 | 0.05 | 0.62±0.08 |
| Ox | | Co | |
| 0.1 | 0.19±0.005 | 0.001 | 0.01±0.01 |
| 1 | 0.61±0.04 | 0.01 | 0.42±0.11 |
| 10 | 0.88±0.02 | 0.1 | 0.87±0.03 |
| TH1 | | YH12 | |
| 0.1 | 0.07±0.003 | 0.5 | 0.07±0.003 |
| 1 | 0.49±0.03 | 5 | 0.37±0.001 |
| 10 | 0.85±0.03 | 50 | 0.93±0.03 |

When used in combination

| Drug conc. (μM) | | Time schedule | | | | |
|---|---|---|---|---|---|---|
| | | 0/0 h | 4/0 h | 0/4 h | 24/0 h | 0/24 h |
| | | Effect | Effect | Effect | Effect | Effect |
| Cs | Tx | | | | | |
| 0.250 | 0.00025 | 0.36±0.01 | 0.68±0.05 | 0.39±0.02 | 0.37±0.01 | 0.41±0.03 |
| 2.500 | 0.00250 | 0.74±0.08 | 0.85±0.05 | 0.61±0.06 | 0.66±0.003 | 0.70±0.06 |
| 25.000 | 0.02500 | 0.83±0.03 | 0.93±0.02 | 0.83±0.04 | 0.81±0.05 | 0.88±0.05 |
| Cs | Co | | | | | |
| 0.250 | 0.0005 | 0.30±0.03 | 0.67±0.06 | 0.42±0.09 | 0.53±0.03 | 0.47±0.03 |
| 2.500 | 0.0050 | 0.76±0.07 | 0.82±0.04 | 0.74±0.04 | 0.59±0.02 | 0.83±0.01 |
| 25.000 | 0.0500 | 0.91±0.02 | 0.92±0.02 | 0.92±0.02 | 0.82±0.08 | 0.88± 0.04 |
| Ox | Tx | | | | | |
| 0.05 | 0.00025 | 0.17±0.01 | 0.29±0.02 | 0.21±0.005 | 0.36±0.02 | 0.21±0.01 |
| 0.5 | 0.0025 | 0.74±0.04 | 0.83±0.03 | 0.73±0.05 | 0.77±0.01 | 0.69±0.05 |
| 5 | 0.025 | 0.90±0.04 | 0.92±0.02 | 0.92±0.02 | 0.85±0.03 | 0.85±0.02 |
| Ox | Co | | | | | |
| 0.05 | 0.0005 | 0.13±0.09 | 0.68±0.04 | 0.24±0.002 | 0.61±0.02 | 0.26±0.02 |
| 0.5 | 0.005 | 0.58±0.06 | 0.76±0.03 | 0.63±0.04 | 0.73±0.05 | 0.66±0.04 |
| 5 | 0.05 | 0.78±0.09 | 0.85±0.03 | 0.85±0.02 | 0.84±0.06 | 0.81±0.04 |
| TH1 | Tx | | | | | |
| 0.05 | 0.00025 | 0.12±0.01 | 0.16±0.002 | 0.26±0.02 | 0.13±0.02 | 0.11±0.01 |
| 0.5 | 0.0025 | 0.60±0.03 | 0.71±0.03 | 0.53±0.03 | 0.72±0.05 | 0.24±0.01 |
| 5 | 0.025 | 0.89±0.02 | 0.90±0.03 | 0.88±0.03 | 0.88±0.01 | 0.85±0.05 |
| TH1 | Co | | | | | |
| 0.05 | 0.00025 | 0.10±0.01 | 0.56±0.05 | 0.27±0.02 | 0.52±0.02 | 0.38±0.01 |
| 0.5 | 0.0025 | 0.39±0.04 | 0.61±0.04 | 0.48±0.03 | 0.57±0.01 | 0.49±0.04 |
| 5 | 0.025 | 0.82±0.03 | 0.85±0.02 | 0.83±0.01 | 0.84±0.06 | 0.83±0.02 |
| YH12 | Tx | | | | | |
| 0.25 | 0.00025 | 0.16±0.01 | 0.27±0.02 | 0.33±0.03 | 0.44±0.04 | 0.34±0.02 |
| 2.5 | 0.0025 | 0.44±0.01 | 0.74±0.004 | 0.53±0.01 | 0.55±0.04 | 0.46±0.004 |
| 25 | 0.025 | 0.84±0.02 | 0.89±0.03 | 0.88±0.04 | 0.82±0.05 | 0.83±0.04 |
| YH12 | Co | | | | | |
| 0.25 | 0.0005 | 0.10±0.01 | 0.47±0.01 | 0.27±0.02 | 0.60±0.001 | 0.32±0.02 |
| 2.5 | 0.005 | 0.29±0.01 | 0.51±0.02 | 0.32±0.01 | 0.59±0.02 | 0.40±0.01 |
| 25 | 0.05 | 0.86±0.04 | 0.85±0.02 | 0.80±0.04 | 0.75±0.01 | 0.83±0.005 |

Table III. *Dose–effect parameters for the combination between platinum compounds and Tx or Co in A2780$^{cisR}$ cell line.*

When used alone

| Drug conc. (μM) | Effect | Drug conc. (μM) | Effect |
|---|---|---|---|
| Cs | | Tax | |
| 0.5 | 0.09±0.01 | 0.0005 | 0.06±0.005 |
| 5 | 0.44±0.02 | 0.005 | 0.48±0.02 |
| 50 | 0.92±0.03 | 0.05 | 0.85±0.04 |
| Ox | | Col | |
| 0.1 | 0.03±0.002 | 0.001 | 0.13±0.01 |
| 1 | 0.51±0.01 | 0.01 | 0.64±0.01 |
| 10 | 0.71±0.01 | 0.1 | 0.95±0.004 |
| TH1 | | YH12 | |
| 0.1 | 0.09±0.004 | 0.5 | 0.02±0.002 |
| 1 | 0.24±0.02 | 5 | 0.49±0.004 |
| 10 | 0.81±0.03 | 50 | 0.92±0.04 |

When used in combination

| Drug conc. (μM) | | Time schedule | | | | |
|---|---|---|---|---|---|---|
| | | 0/0 h | 4/0 h | 0/4 h | 24/0 h | 0/24 h |
| | | Effect | Effect | Effect | Effect | Effect |
| Cs | Tx | | | | | |
| 0.25 | 0.00025 | 0.16±0.02 | 0.48±0.05 | 0.18±0.01 | 0.28±0.004 | 0.16±0.01 |
| 2.5 | 0.0025 | 0.32±0.01 | 0.70±0.07 | 0.39±0.03 | 0.43±0.02 | 0.44±0.02 |
| 25 | 0.025 | 0.87±0.04 | 0.91±0.02 | 0.90±0.04 | 0.87±0.03 | 0.87±0.03 |
| CS | Co | | | | | |
| 0.25 | 0.0005 | 0.11±0.001 | 0.57±0.06 | 0.36±0.003 | 0.49±0.05 | 0.14±0.01 |
| 2.5 | 0.005 | 0.31±0.02 | 0.61±0.06 | 0.45±0.02 | 0.54±0.06 | 0.43±0.03 |
| 25 | 0.05 | 0.91±0.03 | 0.93±0.01 | 0.91±0.01 | 0.84±0.05 | 0.80±0.04 |
| Ox | Tx | | | | | |
| 0.05 | 0.00025 | 0.06±0.002 | 0.27±0.03 | 0.12±0.001 | 0.20±0.01 | 0.11±0.02 |
| 0.5 | 0.0025 | 0.38±0.02 | 0.53±0.05 | 0.46±0.03 | 0.51±0.01 | 0.55±0.08 |
| 5 | 0.025 | 0.83±0.03 | 0.87±0.04 | 0.81±0.03 | 0.84±0.03 | 0.78±0.02 |
| Ox | Co | | | | | |
| 0.05 | 0.0005 | 0.07±0.004 | 0.52±0.06 | 0.17±0.01 | 0.49±0.03 | 0.23±0.01 |
| 0.5 | 0.005 | 0.43±0.02 | 0.60±0.01 | 0.41±0.001 | 0.54±0.06 | 0.41±0.01 |
| 5 | 0.05 | 0.91±0.04 | 0.92±0.04 | 0.91±0.06 | 0.81±0.06 | 0.70±0.03 |
| TH1 | Tx | | | | | |
| 0.05 | 0.00025 | 0.06±0.001 | 0.26±0.01 | 0.12±0.07 | 0.17±0.01 | 0.02±0.01 |
| 0.5 | 0.0025 | 0.23±0.02 | 0.31±0.03 | 0.23±0.09 | 0.34±0.04 | 0.08±0.03 |
| 5 | 0.025 | 0.83±0.02 | 0.80± 0.01 | 0.77±0.06 | 0.81±0.08 | 0.70±0.09 |
| TH1 | Co | | | | | |
| 0.05 | 0.00025 | 0.03±0.001 | 0.48±0.01 | 0.11±0.05 | 0.42±0.01 | 0.15±0.01 |
| 0.5 | 0.0025 | 0.13±0.002 | 0.52±0.01 | 0.29±0.03 | 0.49±0.03 | 0.23±0.004 |
| 5 | 0.025 | 0.89±0.05 | 0.95±0.01 | 0.95±0.001 | 0.89±0.02 | 0.84±0.02 |
| YH12 | Tx | | | | | |
| 0.25 | 0.00025 | 0.08±0.002 | 0.37±0.02 | 0.19±0.01 | 0.38±0.004 | 0.22±0.02 |
| 2.5 | 0.0025 | 0.18±0.01 | 0.36±0.02 | 0.30±0.02 | 0.41±0.01 | 0.33±0.02 |
| 25 | 0.025 | 0.81±0.02 | 0.82±0.02 | 0.79±0.02 | 0.82±0.03 | 0.72±0.01 |
| YH12 | Co | | | | | |
| 0.25 | 0.0005 | 0.16±0.01 | 0.57±0.02 | 0.20±0.02 | 0.48±0.03 | 0.16±0.01 |
| 2.5 | 0.005 | 0.19±0.01 | 0.58±0.01 | 0.34±0.06 | 0.56±0.03 | 0.36±0.06 |
| 25 | 0.05 | 0.94±0.01 | 0.94±0.01 | 0.94±0.01 | 0.76±0.01 | 0.84±0.06 |

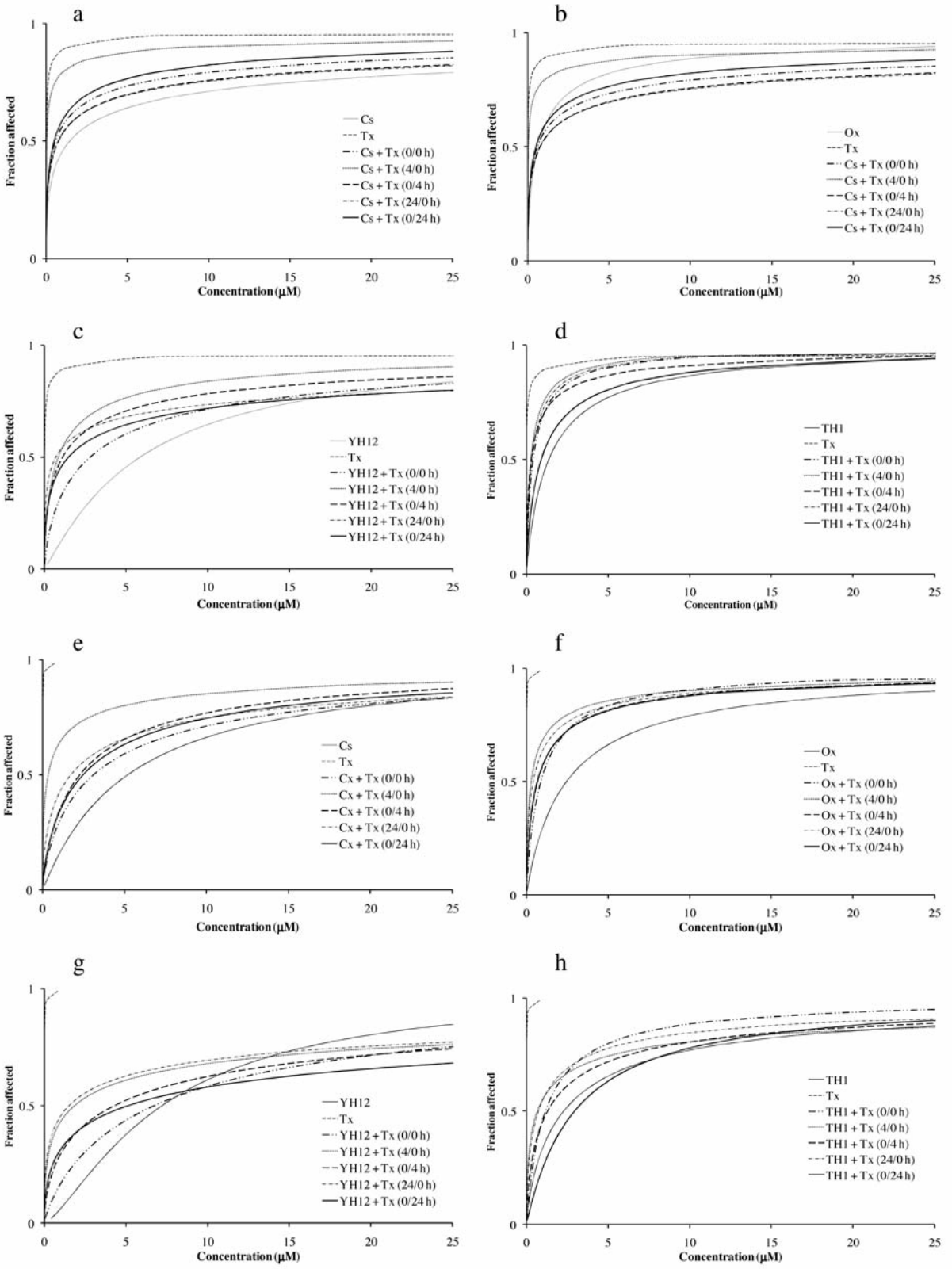


Figure 2. *Dose–response curves for combinations of platinum compounds and Tx in ovarian cancer cell lines: A2780: a) Cs and Tx, b) Ox and Tx, c)YH12 and Tx and d) TH1 and Tx; and A2780$^{cisR}$: e) Cs and Tx, f) Ox and Tx, g) YH12 and Tx and h) TH1 and Tx.*

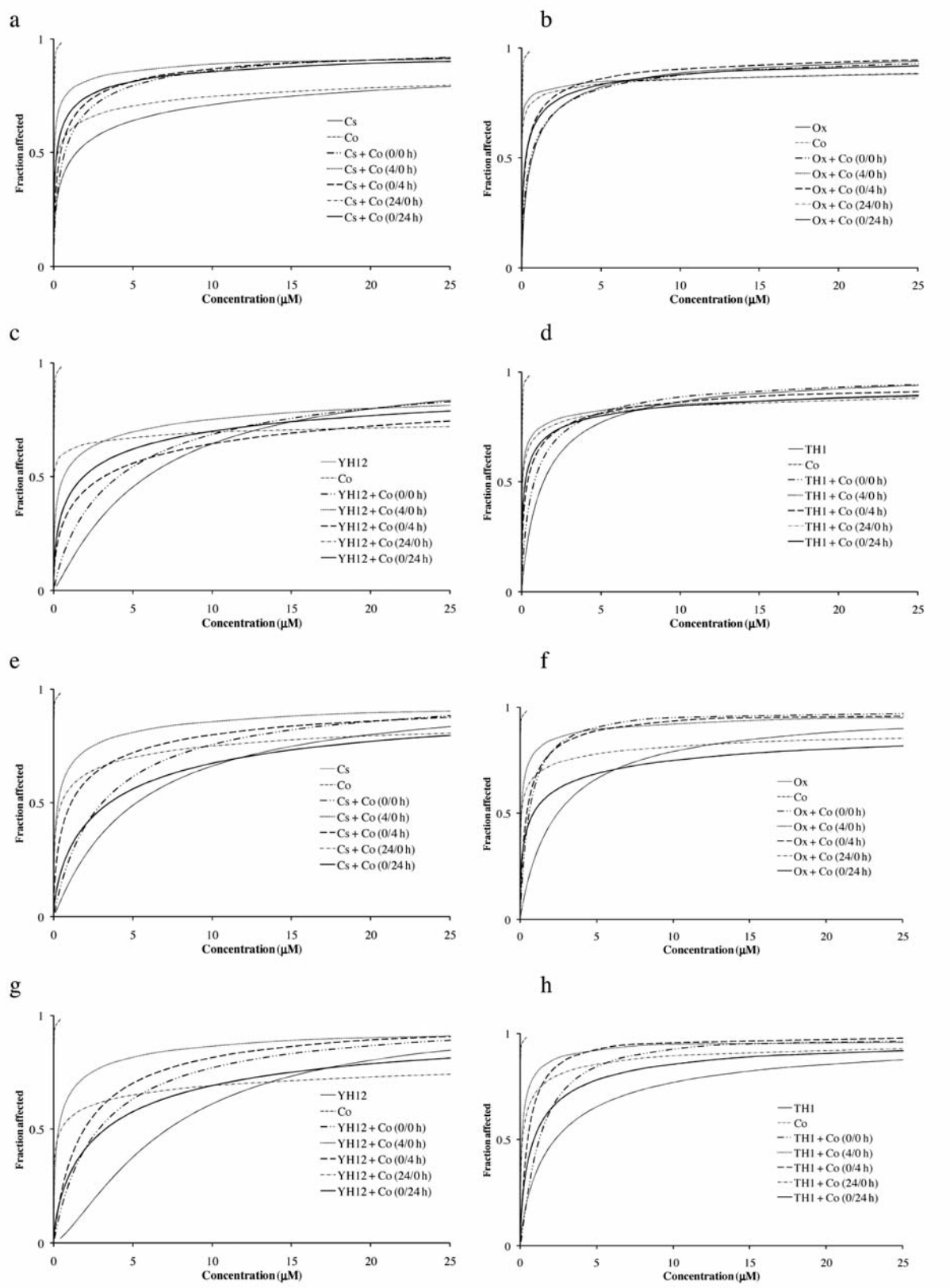


Figure 3. *Dose–response curves for combinations of platinum compounds and Co in ovarian cancer cell lines: A2780: a) Cs and Co, b) Ox and Co, c) YH12 and Co and d) TH1 and Co; and A2780$^{cisR}$: e) Cs and Co, f) Ox and Co, g) YH12 and Co and h) TH1 and Co.*

Table IV. *Dose–effect parameters applying to combinations of platinum compounds with Co and Tx in A2780 and A2780$^{cisR}$ cell lines.*

| Drug | Time sequence | Molar ratio of drugs | A2780 | | | | A2780$^{cisR}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $D_m$ | m | r | $EF_m$ | $D_m$ | m | r | $EF_m$ |
| Cs | | | 1.44 | 0.47 | 1.00 | | 5.18 | 1.03 | 1.00 | |
| YH12 | | | 5.86 | 1.12 | 0.99 | | 7.17 | 1.38 | 0.99 | |
| Ox | | | 0.64 | 0.76 | 1.00 | | 2.45 | 0.93 | 0.94 | |
| TH1 | | | 1.41 | 0.95 | 0.99 | | 2.36 | 0.83 | 0.98 | |
| Co | | | 0.01 | 0.96 | 1.00 | | 0.01 | 0.91 | 0.99 | |
| Tx | | | 0.01 | 0.44 | 0.91 | | 0.01 | 0.97 | 0.99 | |
| Cs+Co | 0/0 h | 500:1 | 0.71 | 0.68 | 0.99 | 1.58 | 3.17 | 0.95 | 0.97 | 0.80 |
| | 4/0 h | | 0.04 | 0.37 | 1.00 | 28.01 | 0.27 | 0.49 | 0.89 | 9.44 |
| | 0/4 h | | 0.44 | 0.61 | 1.00 | 2.55 | 1.09 | 0.64 | 0.92 | 2.34 |
| | 24/0 h | | 0.27 | 0.30 | 0.94 | 4.15 | 0.52 | 0.37 | 0.91 | 4.90 |
| | 0/24 h | | 0.19 | 0.45 | 0.94 | 5.90 | 3.54 | 0.70 | 1.00 | 0.72 |
| Cs+Tx | 0/0 h | 1000:1 | 0.60 | 0.47 | 0.96 | 2.10 | 3.12 | 0.76 | 0.96 | 1.09 |
| | 4/0 h | | 0.04 | 0.39 | 1.00 | 31.50 | 0.35 | 0.51 | 0.99 | 9.76 |
| | 0/4 h | | 0.77 | 0.45 | 1.00 | 1.64 | 2.31 | 0.80 | 0.97 | 1.48 |
| | 24/0 h | | 0.74 | 0.43 | 0.99 | 1.70 | 1.77 | 0.61 | 0.95 | 1.93 |
| | 0/24 h | | 0.50 | 0.52 | 1.00 | 2.52 | 2.44 | 0.77 | 0.99 | 1.40 |
| YH12+Co | 0/0 h | 500:1 | 4.15 | 0.87 | 0.98 | 0.65 | 2.92 | 0.94 | 0.88 | 1.01 |
| | 4/0 h | | 0.67 | 0.40 | 0.90 | 4.03 | 0.31 | 0.54 | 0.88 | 9.52 |
| | 0/4 h | | 3.15 | 0.53 | 0.91 | 0.86 | 1.92 | 0.92 | 0.94 | 1.54 |
| | 24/0 h | | 0.04 | 0.15 | 0.84 | 67.58 | 0.51 | 0.27 | 0.96 | 5.79 |
| | 0/24 h | | 1.87 | 0.51 | 0.92 | 1.45 | 3.26 | 0.71 | 0.98 | 0.91 |
| YH12+Tx | 0/0 h | 1000:1 | 2.82 | 0.73 | 1.00 | 1.31 | 6.66 | 0.86 | 0.96 | 0.63 |
| | 4/0 h | | 0.88 | 0.67 | 0.98 | 4.20 | 1.81 | 0.44 | 0.86 | 2.31 |
| | 0/4 h | | 1.14 | 0.58 | 0.98 | 3.25 | 4.21 | 0.60 | 0.94 | 0.99 |
| | 24/0 h | | 0.69 | 0.39 | 0.96 | 5.36 | 1.51 | 0.44 | 0.90 | 2.77 |
| | 0/24 h | | 1.51 | 0.49 | 0.96 | 2.45 | 5.22 | 0.48 | 0.96 | 0.80 |
| Ox+Co | 0/0 h | 100:1 | 0.57 | 0.69 | 0.98 | 0.69 | 0.61 | 1.05 | 1.00 | 1.45 |
| | 4/0 h | | 0.002 | 0.22 | 0.99 | 197.07 | 0.08 | 0.51 | 0.92 | 11.08 |
| | 0/4 h | | 0.28 | 0.63 | 1.00 | 1.41 | 0.44 | 0.85 | 0.98 | 2.01 |
| | 24/0 h | | 0.01 | 0.26 | 1.00 | 39.41 | 0.10 | 0.32 | 0.91 | 0.80 |
| | 0/24 h | | 0.26 | 0.54 | 0.98 | 1.52 | 0.86 | 0.45 | 1.00 | 9.44 |
| Ox+Tx | 0/0h | 200:1 | 0.26 | 0.81 | 0.97 | 1.87 | 0.88 | 0.93 | 1.00 | 2.34 |
| | 4/0h | | 0.12 | 0.73 | 0.96 | 4.06 | 0.29 | 0.62 | 0.99 | 4.90 |
| | 0/4h | | 0.2 | 0.81 | 0.99 | 2.44 | 0.66 | 0.75 | 1.00 | 0.72 |
| | 24/0h | | 0.11 | 0.51 | 0.96 | 4.43 | 0.43 | 0.66 | 1.00 | 1.09 |
| | 0/24h | | 0.27 | 0.67 | 0.97 | 1.80 | 0.67 | 0.72 | 0.98 | 9.76 |
| TH1+Co | 0/0h | 100:1 | 0.78 | 0.80 | 1.00 | 0.51 | 1.22 | 1.19 | 0.97 | 1.48 |
| | 4/0h | | 0.04 | 0.33 | 0.92 | 9.85 | 0.11 | 0.63 | 0.89 | 1.93 |
| | 0/4h | | 0.37 | 0.57 | 0.99 | 1.07 | 0.52 | 1.08 | 0.96 | 1.40 |
| | 24/0h | | 0.07 | 0.34 | 0.92 | 5.63 | 0.17 | 0.52 | 0.91 | 1.01 |
| | 0/24h | | 0.23 | 0.45 | 0.95 | 1.71 | 0.91 | 0.73 | 0.93 | 9.52 |
| TH1+Tx | 0/0h | | 0.40 | 0.88 | 1.00 | 2.08 | 1.18 | 0.96 | 0.99 | 1.54 |
| | 4/0h | 200:1 | 0.28 | 0.83 | 0.98 | 2.97 | 0.65 | 0.53 | 0.91 | 5.79 |
| | 0/4h | | 0.29 | 0.66 | 0.99 | 2.87 | 1.30 | 0.69 | 0.95 | 0.91 |
| | 24/0h | | 0.33 | 0.85 | 0.97 | 2.52 | 0.74 | 0.66 | 0.97 | 0.63 |
| | 0/24h | | 0.90 | 0.84 | 0.96 | 0.92 | 2.83 | 1.10 | 0.99 | 2.31 |

*Effect of changes in concentration*. For the combinations of Cs with Tx and Co, the effect of changes in concentration on synergism was investigated, in addition to that of the sequence of addition. Figure 4 shows the effect of changes in concentrations on CI values applying to combinations of Cs with Tx and Co in A2780 and A2780$^{cisR}$ cell lines.

It can be seen that the changes in CI values with the change in concentration are more pronounced for the combinations of Cs with Tx in the A2780$^{cisR}$ cell line, and those of Cs with Co in both A2780 and A2780$^{cisR}$ cell lines, indicating pronounced changes on synergism with concentration. The smallest changes in CI with concentration

Table V. *The combination indices at $f_a$=0.5 ($IC_{50}$) of platinum drugs (Cs, Y, Ox, T) and plant drugs (Co, Tx) in ovarian cancer cell lines (A2780 and A2780$^{cisR}$).*

| Plant compound | Platinum compound | Cell line | Combinations for drug schedules | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0/0 h | 4/0 h | 0/4 h | 24/0 h | 0/24 h |
| Tx | Cs | A2780 | 0.48 | 0.03 | 0.62 | 0.59 | 0.40 |
| | | A2780$^{cisR}$ | 1.03 | 0.12 | 0.76 | 0.58 | 0.81 |
| | YH12 | A2780 | 0.80 | 0.25 | 0.32 | 0.20 | 0.43 |
| | | A2780$^{cisR}$ | 1.84 | 0.50 | 1.17 | 0.42 | 1.45 |
| | Ox | A2780 | 0.54 | 0.25 | 0.44 | 0.23 | 0.58 |
| | | A2780$^{cisR}$ | 0.96 | 0.32 | 0.73 | 0.47 | 0.73 |
| | TH1 | A2780 | 0.51 | 0.36 | 0.37 | 0.42 | 1.15 |
| | | A2780$^{cisR}$ | 1.31 | 0.72 | 1.44 | 0.82 | 3.15 |
| Co | Cs | A2780 | 0.60 | 0.03 | 0.37 | 0.23 | 0.16 |
| | | A2780$^{cisR}$ | 1.19 | 0.10 | 0.41 | 0.19 | 1.32 |
| | YH12 | A2780 | 1.31 | 0.21 | 0.99 | 0.01 | 0.59 |
| | | A2780$^{cisR}$ | 0.94 | 0.10 | 0.62 | 0.16 | 1.05 |
| | Ox | A2780 | 1.30 | 0.004 | 0.65 | 0.02 | 0.60 |
| | | A2780$^{cisR}$ | 0.80 | 0.10 | 0.58 | 0.13 | 1.14 |
| | TH1 | A2780 | 1.11 | 0.06 | 0.53 | 0.10 | 0.33 |
| | | A2780$^{cisR}$ | 1.64 | 0.15 | 0.69 | 0.23 | 1.21 |

were generally observed for the combinations of Cs with Tx in the A2780 cell line. Among the five addition sequences, for the combinations of Cs and Tx, CI values for the 0/0 h and 4/0 h additions were found to be least affected by changes in concentration. The CI values for the 4/0 h combination being the lowest, it is clear that for best synergism at all concentrations from combinations of Tx and Cs in the A2780 cell line, Tx should be added first followed by Cs 4 h later. As applied to the combinations of Cs and Tx in the A2780$^{cisR}$ cell line, although the CI value increases with the increasing concentration for all modes of addition, again the 4/0 h addition was found to be associated with the lowest CI values at all concentrations. For the combinations of Cs and Co in the A2780 cell line, the CI values applying to 4/0 h and 0/24 h additions were found to be very low and almost independent of changes in concentration. The results suggest that addition of Co first followed by Cs 4 h later, and the addition of Cs first followed by Co 24 h later produce pronounced synergism in the A2780 cell line at all concentrations. For the combinations of Cs with Co in the A2780$^{cisR}$ cell line, although the CI value changes with concentration for all modes of addition, the 4/0 h addition sequence was associated with the lowest CI values.

*Cell uptake of platinum*. Figure 5 gives the platinum uptakes in A2780 and A2780$^{cisR}$ cell lines applying to the 4/0 h and 24/0 h combinations of Cs with Tx and Co. It was found that the platinum uptakes from 4/0 h and 24/0 h combinations of Cs and Tx were significantly greater than that resulting from

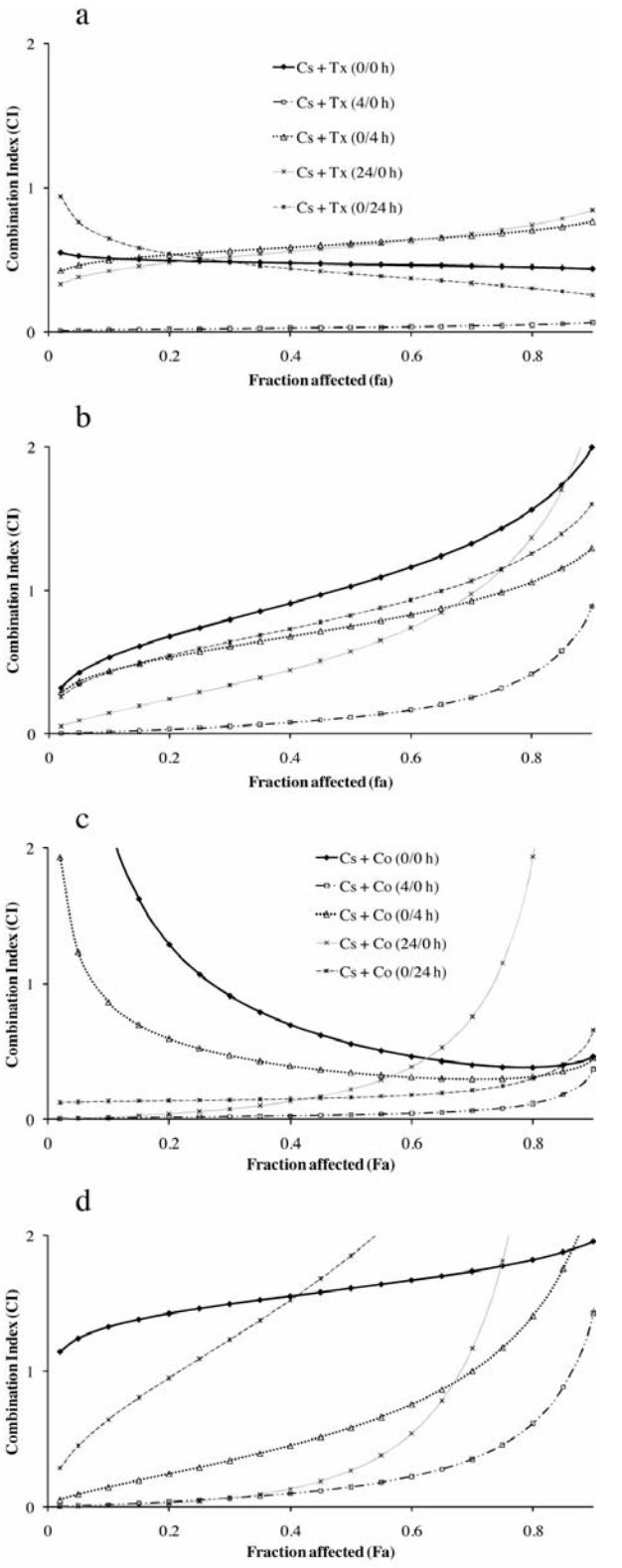


Figure 4. *Combination indices (CIs) versus concentration plots for combinations of Cs with Tx in a) A2780, and b) A2780$^{cisR}$, and combinations of Cs and Co in c) A2780 and d) A2780$^{cisR}$ ovarian cancer cell lines.*

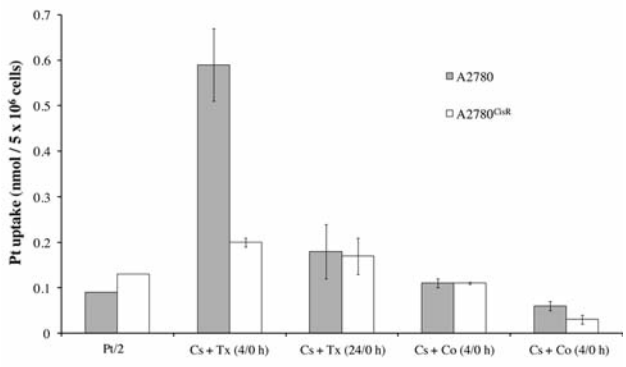


Figure 5. *Total intracellular platinum levels found in the cell lines A2780 and A2780$^{cisR}$.*

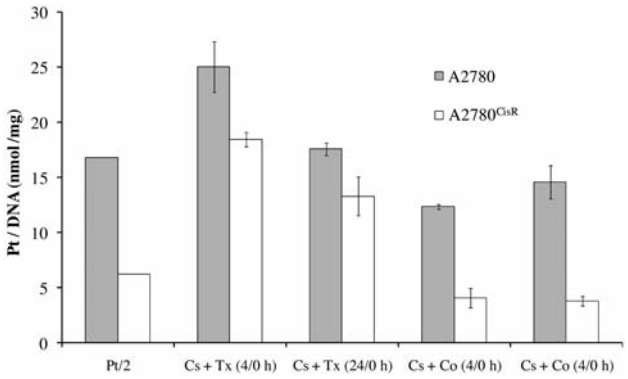


Figure 6. *Platinum-DNA binding levels in A2780 and A2780$^{cisR}$ cell lines applying to the 4/0 h and 24/0 h combinations of Cs with Tx and Co.*

equivalent concentration of cisplatin alone. Generally, platinum uptakes in the A2780 cell line were higher than those in the A2780$^{cisR}$ cell line (except for the 4/0 h combination of Cs and Co where the platinum uptake in the resistant cell line was found to be slightly greater). The sequence 24/0 h was associated with lower platinum uptake than the sequence 4/0 h. Highest platinum uptake was observed for the 4/0 h combination of Cs and Tx in the A2780 cell line and the lowest platinum uptake was observed for the 24/0 h combination of Cs and Co in the A2780$^{cisR}$ cell line. It will be seen later that the highest platinum uptake from the 4/0 h combination of Cs and Tx was also associated with the highest platinum-DNA binding levels.

*Platinum-DNA binding levels*. Figure 6 gives the platinum-DNA binding levels in A2780 and A2780$^{cisR}$ cell lines applying to the 4/0 h and 24/0 h combinations of Cs with Tx and Co. As noted earlier, the highest platinum-DNA binding level was observed for the 4/0 h combination of Cs and Tx for the A2780 cell line and the lowest for the 24/0 h combination of Cs and Co for the A2780$^{cisR}$ cell line (in line with the lowest cell uptake of platinum). For the 4/0 h combination of Cs with Tx, platinum-DNA binding level in the A2780$^{cisR}$ cell line was also found to be high. The platinum-DNA binding level in the A2780 cell line applying to the 24/0 h combination of Cs and Co was found to be quite high even though the corresponding cell uptake of platinum was low.

## Discussion

It can be seen that for the combination of Cs with Tx, generally greater synergism was observed at lower concentrations than at higher concentrations and more so in the resistant cell line A2780$^{cisR}$ than in the parent cell line. As applied to the combination of Cs and Co, the situation appears to be more complex. Whereas in the resistant cell line, the observed synergism decreased with increasing concentration, in the parent A2780 cell line, the observed synergism increased with increasing concentration for 0/4 h and 4/0 h modes of addition; it decreased (*i.e.* CI value increased) for the 0/24 h mode of addition and was essentially independent of changes in concentration for the 4/0 h mode of addition.

From the above, it is clear that both Tx and Co produce sequence- and concentration-dependent synergism with Cs, Ox, YH12 and TH1. If these results are found to be true *in vivo*, they would have profound implications in the design of combination therapy. For example, high synergism observed in 4/0 h and 24/0 h modes of addition but antagonism observed in the 0/0 h, 0/4 h and 0/24 h modes of addition indicate that the administration of platinum drug in bolus with the phytochemical or 4 h and 24 h before the administration of Tx or Co would have no therapeutic advantage, rather it may be counterproductive. On the other hand, administration of Tx or Co, 4 h or 24 h before the administration of platinum would be associated with therapeutic advantage. Why the administration of platinum followed by that of phytochemicals some time later produces greater synergism is a fundamental question that is likely to be associated with differences in mechanisms of action of the combined drugs. Recently it has been reported that pretreatment of ovarian cancer cells with the flavanoid genistein increased their sensitivity to Cs treatment (26), whereas in the present study the converse was found to be true as applied to the combination of Cs (and other platinum compounds) with Tx and Co. In another study in which synergism was observed in human breast cancer cells *in vitro* between Cs and dipyridamole that has been clinically used as a coronary vasodilator, it was found that whereas the

synergism in Cs-sensitive MDA/S human breast cancer cell was associated with increased platinum accumulation, that in Cs-resistant MDA/R cells did not result in any increase in platinum accumulation (27). The authors suggested that dipyridamole might interact with the cell membrane of MDA/S cells so as to increase the intracellular accumulation of Cs. However, whether the increased accumulation of platinum in MDA/S human breast cancer cell resulted in its increased binding with DNA or not was not determined. In a phase II study of combination of Tx and Ox in 105 patients with platinum-sensitive recurrent advanced ovarian cancer who received 175 mg/m$^2$ Tx (over 3 h) followed by 130 mg/m$^2$ Ox (over 2 h) every 21 days up to nine cycles without prior hydration, significant synergism in activity was observed (28).

As platinum drug-resistance in ovarian cancer cell lines may be associated with reduced cell uptake, increased deactivation of the drug before binding with DNA and increased DNA repair, it is important to determine whether combination of platinum compounds with phytochemicals has an effect on cell uptake and level of platinum binding with DNA. If so, the results may partly explain the occurrence of synergism from combination of cisplatin with phytochemicals. It may be noted that although copper transporter 1 protein (CTR1) is a major carrier protein for the transport of copper into the cell, Cs itself triggers down-regulation and proteasomal degradation of CTR1, thereby limiting its own uptake.

It was found that the platinum uptakes from 4/0 h and 24/0 h combinations of Cs and Tx were significantly greater than that resulting from equivalent concentration of cisplatin alone (Figure 5). The results show that the addition of Tx before the addition of Cs serves to increase the cellular uptake of Cs. The increase in cellular uptake of Cs due to combination with Tx may be compared with that due to combination with bortezomib. It has been reported that bortezomib, when administered *i.p.* 4 h before *i.p.* injection of Cs enhanced the delivery of Cs to intraperitoneal ovarian carcinomas in female *nu/nu* mice (30).

When uptakes in A2780 and A2780$^{cisR}$ cell lines due to Cs alone are compared, it is found that the platinum uptake in the A2780 cell line was lower than that in the A2780$^{cisR}$ cell line, indicating that reduced uptake is not a likely mechanism of resistance in the A2780$^{cisR}$ cell line. The sequence 24/0 h was associated with lower platinum uptake than the sequence 4/0 h. This is to be expected simply from the difference in period of incubation with Cs.

It can be seen that the highest platinum-DNA binding level observed for the 4/0 h combination of Cs and Tx in the A2780 cell line is in line with the highest platinum cell uptake and the lowest platinum-DNA binding level observed for the 24/0 h combination of Cs and Co in the A2780$^{cisR}$ cell line is in line with the lowest platinum cell uptake (Figure 6). For the 4/0 h combination of Cs with Tx, platinum-DNA binding level in the A2780$^{cisR}$ cell line also, is found to be high in line with high platinum cell uptake and high synergism. The platinum-DNA binding level in the A2780 cell line applying to the 24/0 h combination of Cs and Co was quite high even though the corresponding cell uptake of platinum was low. The results may suggest that the addition of Co 24 h before that of Cs has the effect of reducing DNA repair, which is a likely mechanism of platinum resistance in ovarian cancer cells. When the period of incubation is extended to 72 h, in the absence of further information, whether the observed difference in platinum-DNA binding levels associated with the 4/0 h and 24/0 h additions of Cs and Co in the A2780 cell line may persist or not remains an open question.

The results of the present study show that combination of selected platinum compounds Cs, Ox, YH12 and TH1 with phytochemicals Tx and Co show both concentration- and sequence-dependent synergism in human ovarian cancer cell lines A2780 and A2780$^{cisR}$. The most significant finding is that the administration of Tx or Co, 4 h or 24 h before the addition of platinum, was associated with high synergism, whereas the converse and the bolus addition were generally associated with antagonism. The results may have profound implications in therapy, if found to be true *in vivo*. Proteomics studies should be carried out to probe the changes in expression of key proteins associated with resistance or overcoming of the same from drug combinations.

## Conflict of interest

Nurhanan M. Yunos, Philip Beale, Jun Qing Yu, Dana Strain and Fazlul Huq declare that they have no financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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