Synergism from Combination of Cisplatin and a *Trans*-Platinum Compound in Ovarian Cancer Cell Lines

ALI ALSHEHRI[1], PHILIP BEALE[2], JUN QING YU[1] and FAZLUL HUQ[1]

[1]*Discipline of Biomedical Science, The University of Sydney, Lidcombe Campus C42, Lidcombe, NSW 1825, Australia;*
[2]*Sydney Cancer Centre, Sydney, NSW, Australia*

Abstract. *Development of drug resistance and the presence of dose-limiting side-effects remain two major problems in cancer chemotherapy. Combination of drugs can offer a means of overcoming drug resistance and reducing side-effects. This study investigated synergism in activity from the combinations of cisplatin (Cis) and trans-planaramineplatinum(II) compound YH12 in the human ovarian A2780, A2780^{cisR} and A2780^{0473R} cancer cell lines. It was found that Cis and YH12 in combination produced both sequence- and concentration-dependent synergism. Addition of Cis first followed by YH12 4 h later produced least synergistic outcomes in all the three cell lines whereas the addition of YH12 first followed by Cis 4 h later and the bolus addition produced much greater synergism. It is believed that as Cis binds with a DNA strand forming intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts, the DNA strand is bent. As a result, the subsequent interstrand bifunctional binding of YH12 to the bent DNA would be hampered due to a greater distance mismatch between the two trans-arms of YH12 and the distance between 1,2-interstrand N7(G) and N7(G) positions especially those close to the cisplatin-binding site. This may explain why the 0/4 h addition would be least synergistic in outcome. Conversely, although the interstrand GG binding of YH12 first (that would occur in the 4/0 h and 0/0 h additions), brings about global changes in DNA conformation, it will not significantly affect the subsequent intrastrand bifunctional binding of Cis, so that these modes of addition would result in greater synergistic outcomes. The results of the present study will have implications in the design of combination therapy if confirmed in vivo.*

Correspondence to: Fazlul Huq, Discipline of Biomedical Science, School of Medical Sciences, Sydney Medical School, The University of Sydney, Cumberland Campus C42, 75 East Street, Lidcombe, NSW 1825, Australia. Tel: +61 293519522, Fax: +61 293519520, e-mail: fazlul.huq@sydney.edu.au

Combination of drugs with different modes of action can offer a distinct advantage over monotherapy in overcoming drug resistance and reducing side-effects (1-3). Although the anticancer activity of cisplatin (Cis) and *trans*-planaramineplatinum(II) complexes is believed to be associated with their binding with DNA, they differ in the spectrum of adducts formed. Whereas Cis and its analogues form mainly intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts with DNA that cause mainly the local bending of a DNA strand, *trans*-planaramineplatinum(II) complexes form mainly interstrand Pt(GG) adducts that would cause more of a global change in the DNA conformation. It is believed that combination of Cis with *trans*-planaramineplatinum(II) complexes may display synergism in activity as the repair enzymes may fail to recognize the adducts formed by *trans*-planaramineplatinum(II) complexes. A number of mononuclear *trans*-planaramineplatinum(II) complexes of the forms *trans*-$[Pt(NH_3)LCl_2]$ and *trans*-$[PtL_2Cl_2]$ where L is a planaramine ligand have been previously designed. One complex of the form *trans*-$[Pt(NH_3)LCl_2]$ where L is (1,2-α)pyridine (coded as YH12) is found to be significantly more active than Cis against the ovarian A2780^{cisR} cancer cell line (4, 5). YH12 has a resistance factor (defined as the concentration of the drug required for 50% cell kill in the resistant cell line over that in the parent cell line) of 0.53 as applied to the A2780 and A2780^{cisR} cell lines whereas Cis has a value of approximately 9. This study investigated synergism in activity from the combination of Cis and YH12 in the human ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cancer cell lines as a function of both the sequence of addition and the changing concentration.

Materials and Methods

Materials. Cis and YH12 were prepared according to previously described methods (4, 6). Foetal calf serum (FCS), RPMI 1640, 20 mM L-glutamine and 5.6% dodium bicarbonate were obtained from Trace Biosciences Pty Ltd Australia. Other chemicals were purchased mostly from Sigma-Aldrich Sydney Australia. Stock solutions of platinum compounds (1 mM) were prepared in 1:1 (DMF)-mQ water mixture.

Cell culture. Human ovarian cancer lines A2780, A2780cisR, and A2780ZD0473R were gifts from Dr. Mei Zhang, Royal Prince Alfred Hospital, Sydney, NSW, Australia. The A2780 cell line was originally established from tumour tissue of an untreated patient (7); the A2780cisR cell line has been developed by chronic exposure of the A2780 cells to increasing concentrations of Cis (8, 9); and the A2780ZD0473R cell line was developed by *in vitro* exposure of A2780 cells to increasing concentrations of the drug ZD0473 from 0.5 to 12.5 μM of for a period of seven months (10). All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

MTT reduction assay. Cytotoxicity was determined using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] reduction assay (11). Between 5000 and 9000 cells were seeded into the wells of the flat-bottomed 96-well culture plate in 10% FCS/RPMI 1640 culture medium. The plate was incubated for 24 h at 37°C in a humidified atmosphere to allow cells to attach. Cis and YH12, dissolved first in a minimum volume of DMF, were diluted to the required concentrations by adding mQ water and filtered to sterilize. Serial fivefold dilutions of the drugs ranging from 0.02 to 62.5 μM in 10% FCS/RPMI-1640 medium were prepared and added to equal volumes of cell culture in quadruplicate wells, left to incubate under normal growth conditions for 72 h. Four hours after the addition of MTT (50 μl per well of 1 mg ml^{-1} MTT solution), formazan crystals produced by live cells were dissolved in 150 ml of DMSO and read with a plate reader set at 570 nm. The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments.

Combination studies. Cells were treated with solutions of Cis and YH12 alone and in combination at three different concentrations. Three modes of addition 0/0 h, 0/4 h and 4/0 h were employed, where 0/0 h indicates that both the compounds were added at the same, 0/4 h means that Cis was added first followed by YH12 4 h later and 4/0 h means that Cis was added 4 h after the addition of YH12. The period of incubation was 72 h counted from the time of addition of the first compound. Inhibition of cell growth was determined using the MTT reduction assay (11). The results were analysed using the program CalcuSyn (12-14) to obtain dose–response curves and combination index (CI) values as measures of synergism, additiveness or antagonism. Each experiment was repeated at least three times. The combination index (CI) for binary combination of drugs was calculated according to the following equation:

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

where D_1 and D_2 in the numerator represent mean doses of compounds 1 and 2 in combination required to cause x% inhibition, respectively, whereas D_{1x} and D_{2x} in the denominator represent the doses of compounds 1 and 2 required to cause x% inhibition when present alone. Dx 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 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 <1, =1 and >1 indicates respectively synergism, additivity and antagonism in combined drug action.

Platinum cellular accumulation and platinum-DNA binding. Cellular accumulations of platinum and platinum-DNA binding levels were measured in order to determine whether the values were affected by the sequence of addition and if so the results may elucidate the relationship between synergism and the sequence of addition. It may be noted that the mediated transport of Cis into the cell by copper transporter CRT1 is limited by Cis itself as it triggers the down-regulation and proteosomal degradation of CTR1 (15).

Cellular accumulation. Platinum complexes (at 50 μM final concentration) were added to culture plates containing exponentially growing A2780 and A2780cisR cells; cells containing the drugs were incubated for 4 h at the end of which cell monolayers were trypsinized and cell suspension (10 ml) was transferred to a centrifuge tube and spun at 3500 rpm for 2 min at 4°C. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at –20°C until assayed. At least three independent experiments were performed. Following incubation with compounds, cell pellets were suspended in 0.5 ml 1% Triton-X, held on ice then sonicated. Total intracellular platinum contents were determined by graphite furnace AAS.

Platinum-DNA binding levels. Following drug incubation high molecular weight DNA was isolated from cell pellet using HPP0050 JETQUICK Blood DNA Spin Kit/50 from Austral Scientific Pty Ltd., Sydney, Australia, and the modified protocol of Bowtell (16). The cell pellets resuspended in PBS to a final volume of 200 μl and mixed with 10 μl of RNase A, were incubated for 4 min at 37°C. Approximately 25 μl proteinase K and 200 μl buffer K1 (containing guanidine hydrochloride and a detergent) were added to the mixture followed by incubation for 10 min at 70°C. Then, 200 μl of absolute ethanol was added and mixed thoroughly to prevent any precipitation of nucleic acids. The samples were centrifuged for 1 min at 10,600 rpm through the silica membrane using JETQUICK micro-spin column from Austral Scientific Pty Ltd Sydney Australia. The columns containing the samples were washed with 500 μl of buffer KX (containing high-salt buffer to remove residual contaminations) and centrifuged for 1 min at 10,600 rpm. These were again washed with 500 μl 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. To further clear the silica membrane from residual liquid, the sample columns were centrifuged again for 2 min at full speed (13,000 rpm). The column receivers were changed and the purified DNA in the column was eluted from the membrane with 200 μl of 10 mM Tris–HCl buffer (pH 8.5). DNA content was determined by UV spectrophotometry (260 nm) and platinum levels were determined by graphite furnace AAS.

Results

Cytotoxicity. Table I gives IC_{50} values and resistance factors (RF) for Cis and YH12, as applied to the ovarian cancer cell lines A2780, A2780cisR, and A2780ZD0473R. RF is defined as the ratio of concentration of the drug required for 50% cell kill in the resistant cell line to that in the parent cell line.

Table I. *IC_{50} values and resistance factors (RF) for Cis and YH12 as applied to the ovarian cancer cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}. RF is defined as the ratio of concentration of the drug required for 50% cell kill in the resistant cell line to that in the parent cell line.*

Compound	IC_{50} values (μM) and resistance factors				
	A2780	A2780^{cisR}		A2780^{ZD0473R}	
	IC_{50}	IC_{50}	RF	IC_{50}	RF
Cis	0.43	3.69	8.68	1.52	3.53
YH12	4.38	2.91	0.67	6.05	1.38

It can be seen that although YH12 is less active than Cis against the parent A2780 cell line, it is more reactive than Cis against the resistant A2780^{cisR} cell line, so that YH12 has a much lower RF than Cis. Whereas Cis binds with DNA forming mainly intrastrand bifunctional 1,2-Pt(GG) and 1,2-Pt(AG) adducts, YH12 is expected to bind with DNA forming mainly interstrand 1,2-Pt(GG) adduct. It is possible that the repair enzymes in the A2780^{cisR} cell line may fail to recognize the interstrand adducts formed by YH12.

Combination studies. Both dose–effect curves and CIs were used to determine synergism from the combination of Cis with YH12. Whereas dose–effect curves gave a visual representation of the combined drug action that was qualitative in nature, CIs gave more of a quantitative measure of the synergism.

A2780 cell line. Figure 1 gives the dose–effect curves for Cis, YH12 and their combinations applying to the 0/0 h, 0/4 h and 4/0 h sequences of addition in the human ovarian A2780 cell line.

It can be seen that for the combination of YH12 and Cis in the A2780 cell line, the greatest synergism was produced when YH12 was added first followed by Cis 4 h later and the least synergism resulted when Cis was added first followed by YH12 4 h later (especially at higher concentrations).

A2780^{CisR} cell line. Figure 2 gives the dose–effect curves for Cis, YH12 and their combinations applying to the 0/0 h, 0/4 h and 4/0 h sequences of addition in the A2780^{CisR} cell line.

It can be seen that for the combinations of YH12 and Cis in the A2780^{cisR} cell line, the greatest synergism was produced when YH12 was added first followed by Cis 4 h later and the least synergism was produced when Cis was added first followed by YH12 4 h later. The results are to a great extent similar to those found in the parent cell line A2780.

A2780^{ZD0473R} cell line. Figure 3 gives the dose–effect curves for Cis, YH12 and their combinations applying to the 0/0 h, 0/4 h and 4/0 h sequences of addition in the A2780^{ZD0473R}.
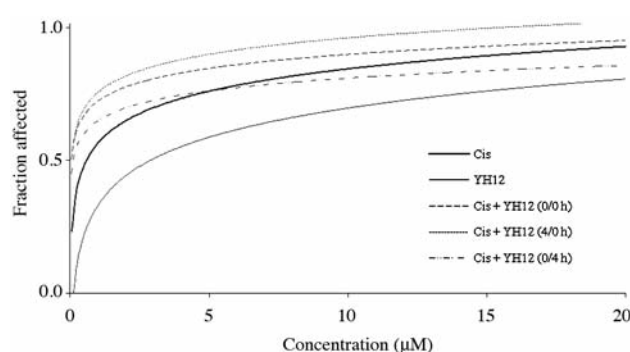

Figure 1. *Dose–effect curves for Cis and YH12 and their combinations applying to the 0/0 h, 4/0 h and 0/4 h sequences of addition in the A2780 cell line.*

It can be seen that for the combinations of Cis and YH12 at low concentrations, greatest synergism in the A2780^{ZD0473R} cell line greatest synergism was produced when YH12 was added first followed by Cis 4 h later. At higher concentrations it was the 0/0 h combination that produced greater synergism. At still higher concentrations, the 0/4 h combination also was found to be more synergistic than the 4/0 h combination. The results are discussed more fully in the discussion section.

Combination index (CI). As stated earlier, besides dose–effect curves, the combined drug action was also analysed using the CIs. Table II gives the CIs at ED_{50}, ED_{75} and ED_{90} for the combinations of Cis and YH12 in the human ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines where ED_{50}, ED_{75} and ED_{90} stand for the combined drug concentrations required for 50%, 75% and 90% cell kill-respectively.

It can be seen that for the combinations of Cis and YH12 in the A2780 cell line, 4/0 h and 0/0 h sequences of addition had the lower CIs whereas the 0/4 h mode of addition had higher CIs. In the A2780^{cisR} and A2780^{ZD0473R} also, the 4/0 h
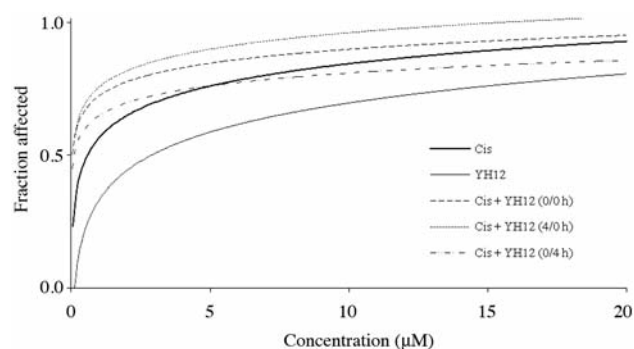
Figure 2. *Dose–effect curves for Cis, YH12 and their combinations applying to the 0/0 h, 0/4 h and 4/0 h sequences of addition in the A2780^{cisR} cell line.*

Figure 3. *Dose–effect curves for Cis, YH12 and their combinations applying to the 0/0 h, 0/4 h and 4/0 h sequences of addition in the A2780^{ZD0473R} cell line.*

and 0/0 h modes of addition were associated with the lower CIs. The results are largely similar to those inferred from dose–effect curves.

Platinum cell uptake. Figure 4 gives the platinum accumulation in the A2780 and A2780^{cisR} cell lines in 4 h from Cis and YH12 and their 0/0 h, 4/0 h and 0/4 h combinations.

It can be seen that in both the A2780 and A2780^{cisR} cell lines, the cellular accumulation of platinum from YH12 alone was much greater than that from Cis alone. It should also be noted that platinum accumulation in the resistant cell line A2780^{cisR} cell line was greater than that in the parent A2780 cell line. As applied to the combinations of Cis and YH12, the 4/0 h combination was associated with the highest platinum accumulation whereas the 0/4 h combination resulted in the lowest value. It should also be noted that the platinum accumulation from the 4/0 h combination was greater than the value expected from uptakes of Cis and YH12. Another point to note is that for the all the three sequences of addition the accumulation of platinum in the resistant cell line was greater than that in the parent cell line. The order of platinum accumulation can be seen to be seen to be in line with that of synergism. The results are discussed further later.

DNA binding. Figure 5 gives levels of platinum-DNA binding in 4 h in the A2780 and A2780^{cisR} cell lines from Cis and YH12 and their 0/0 h, 0/4 h and 4/0 h combinations.

It was found that in both the cell lines, the 0/0 h and 4/0 h sequences of addition were associated with higher platinum-DNA binding levels and 0/4 h combination was associated with lower platinum-DNA binding levels. These results are in line with the order of synergism observed for the three sequences of administration. The highest platinum-DNA binding level observed for the 0/0 h mode of addition (in A2780 cell line) was to be expected as the incubation period was a maximum for the two compounds.
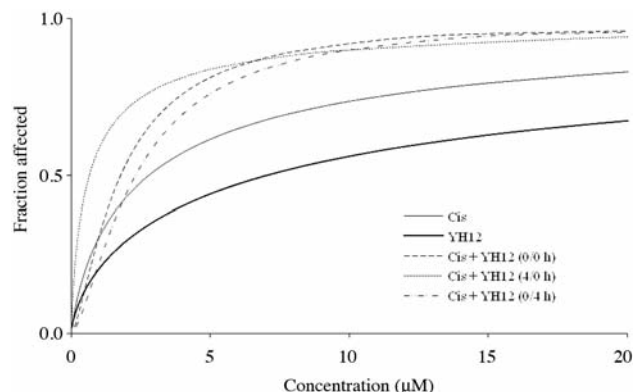
Table II. *CIs for various combinations of Cis and YH12 in the human ovarian A2780, A2780^{cisR} and A2780^{ZD0473R} cell lines.*

Mode of addition	Cell line	CI at		
		ED$_{50}$	ED$_{75}$	ED$_{90}$
0/0 h	A2780	0.38	0.46	1.01
	A2780^{cisR}	0.44	0.34	0.39
	A2780^{ZD0473R}	0.89	0.47	0.24
4/0 h	A2780	0.22	0.36	1.01
	A2780^{cisR}	0.42	0.21	0.16
	A2780^{ZD0473R}	0.32	0.31	0.29
0/4 h	A2780	0.36	1.39	1.24
	A2780^{cisR}	1.17	0.75	0.72
	A2780^{ZD0473R}	1.16	0.58	0.29

Discussion

In this study synergism in activity from the combinations of Cis with a *trans*-planaraminrplatinum(II) compound YH12 in the human ovarian A2780, A2780^{cisR} and A2780^{0473R} cancer cell lines was investigated as a function of both the sequence of administration and the changing concentrations. Both the dose–effect curves and CIs indicated that in all the three cell lines greater synergism was produced from the 4/0 h and 0/0 h combinations than the 0/4 h combination. The results indicate that the sequence specificity of binding with DNA plays a critical role in the activity of platinum drugs. Because YH12 (besides forming monofunctional adducts) can form short-range interstrand adducts such as 1,2-interstrand Pt(GG) adduct (rather than intrastrand adducts), it follows that the bending of a DNA strand due to the intrastrand bifunctional binding of Cis with DNA (17) would render it more difficult
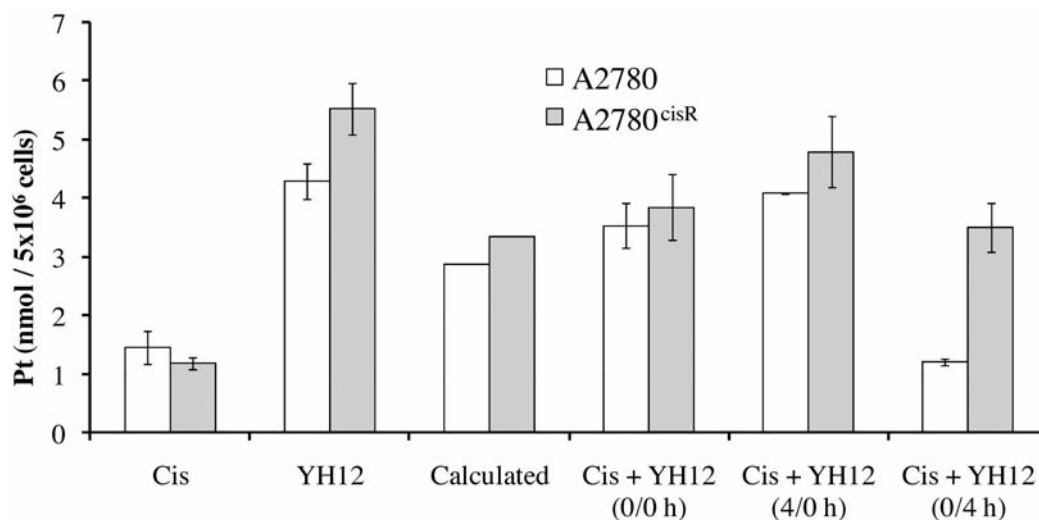
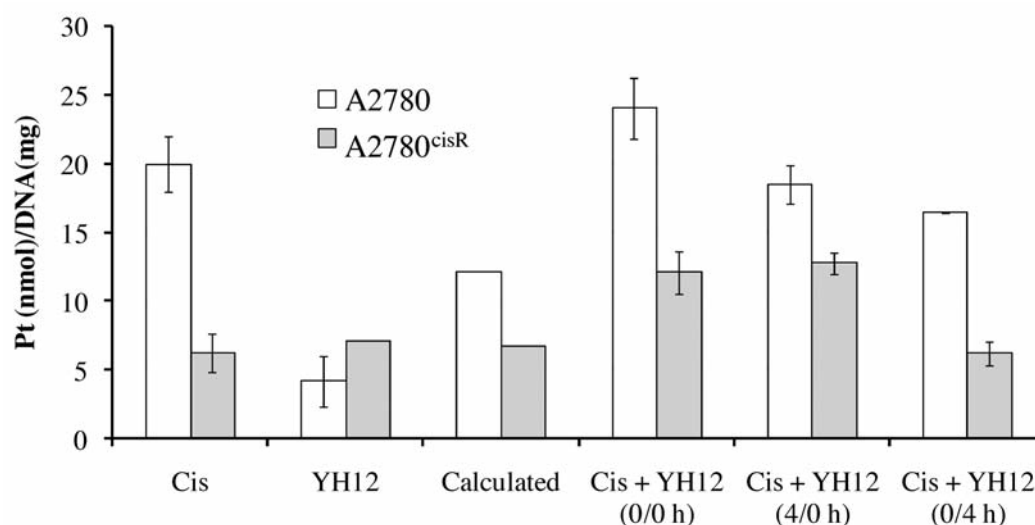Figure 4. *Platinum accumulation in the A2780 and A2780^cisR cell lines from Cis and YH12 and their combinations.*

Figure 5. *Levels of platinum-DNA binding in the A2780 and A2780^cisR cell lines from Cis and YH12 and their combinations.*

for the formation of subsequent interstrand adduct by YH12 (especially at positions close to the Cis bending sites). In other words, the bending of a DNA strand due to its binding with Cis would produce a greater mismatch between interstrand separation and the distance between the two *trans* 'arms' of YH12. Conversely the formation of interstrand bifunctional 1,2-Pt(GG) adduct by YH12 (although it would induce a global distortion of DNA) would not affect the formation of subsequent interstrand 1,2-Pt(GG) adduct by Cis. This is illustrated in Figure 6. Proteomic studies determining differential expressions of key proteins in the parent and the resistant cell lines may provide further insight on the matter.

Cellular accumulations and drug-DNA binding levels. Platinum drugs such as Cis are expected to cross the cell membrane by both passive diffusion and carrier mediated transport and the same may be true for the *trans*-platinum compound YH12 although the actual carriers may or may not be the same. Once inside the cell, platinum drugs may undergo deactivation due to their binding with the cellular platinophiles such as glutathione and metallothionein before binding with the DNA (19). Thus, the cellular accumulation of platinum *per se* may not correspond to the platinum-DNA binding level and the drug activity. It was found that in both the A2780 and A2780^cis cell lines, for the 0/0 h and 4/0 h
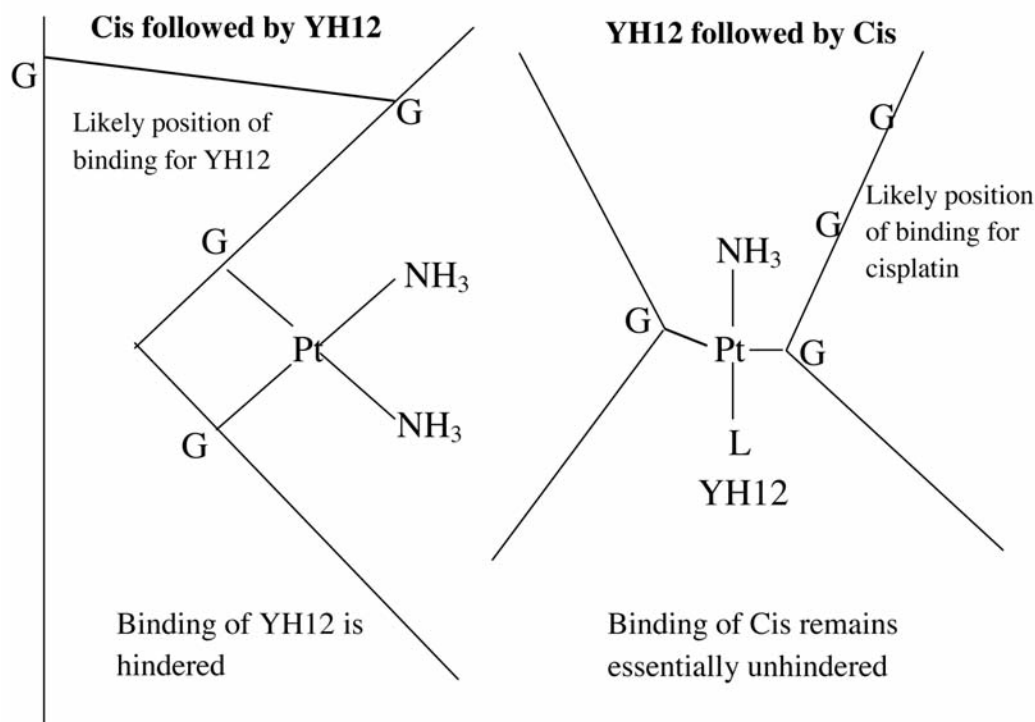
Figure 6. *Simplified representation showing the bending of a DNA strand due to the formation of intrastrand bifunctional 1,2-Pt(GG) adduct by Cis and global distortion of DNA due to the formation of interstrand bifunctional 1,2-Pt(GG) adduct by YH12.*

sequences of addition, the accumulations of platinum were greater than the calculated values (based on the levels observed when the compounds were present alone), whereas for the 0/4 h sequence of addition the value was much lower. The order of cellular accumulation of platinum can be seen to be in line with that of synergism.

The lower cellular accumulation of platinum observed from the 0/4 h sequence of addition may be due to the down-regulation and proteasomal degradation of CTR1 by Cis, thereby limiting its own uptake (15). Another reason for the lower platinum accumulation from 0/4 h combination and higher platinum accumulations from the 0/0 h and 4/0 h combinations is associated with differences in the periods of incubation of the two compounds that differ greatly in their cellular uptake. However, this consideration cannot explain why the cellular accumulation of platinum from the 4/0 h mode of addition was greater than that from the 0/4 h mode of addition.

It was found that in the A2780 and A2780^{cisR} cell lines, the 0/4 h sequence of addition was associated with the lowest level of platinum-DNA binding whereas the 0/0 h sequence of addition was associated with the highest platinum-DNA binding level (especially in the parent A2780 cell line) (Figure 5). Noting that the 0/4 h sequence of addition produced lowest synergistic outcome and that the 0/0 h and

4/0 h sequences of administration resulted in higher synergism, it should be seen that the order of platinum-DNA binding levels was in line with that of synergism. When platinum-DNA binding levels in the parent and resistant cell lines are compared, it can be seen that for all the three modes of addition, the values found in the resistant cell line are lower than those in the parent cell line, thus indicating that DNA repair is a dominant mechanism of platinum resistance operating in the cell lines.

As discussed earlier, the results can also be seen to provide support to the idea that the bending of a DNA strand due to the intrastrand bifunctional binding with Cis would adversely affect the subsequent bifunctional interstrand binding of YH12 with the bent DNA partly due to the distance mismatch.

Conclusion

In this study synergism in activity from the combination of Cis with a "rule-breaking" platinum compound YH12 in the human ovarian A2780, A2780^{cisR} and A2780^{0473R} cancer cell lines was investigated as a function of the sequence of administration and the changing concentrations. The results of the study show that in the A2780, A2780^{cisR} and A2780^{0473R} cell lines, the combined action was more synergistic when

YH12 was added first followed by Cis 4 h later or when the two compounds were added at the same time and least synergistic when Cis was added first followed by YH12 4 h later. If the results are confirmed *in vivo*, these will have clinical significance in the designing of combination chemotherapy for the optimum therapeutic outcomes.

Acknowledgements

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