Synergistic cell inactivation by cis-dichlorodiammineplatinum in combination with 1-propargyl-5-chloropyrimidin-2-one

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Summary

A synergistic effect with respect to inactivation of human NHIK 3025 cells cultured in vitro was displayed when treatment with cis-dichlorodiammineplatinum(II) (cis-DDP) and the mitotic inhibitor 1-propargyl-5-chloropyrimidin-2-one (NY 3170) were given in simultaneous combination. Cell inactivation was measured by loss of colony-forming ability. Treatment with NY 3170 alone produced no significant inactivation at 2 mm. However, treatment with NY 3170 in combination with cis-DDP induced increased cell inactivating effects equal to a doubling of either the concentration of cis-DDP or treatment time. Scheduling of NY 3170 treatment in relationship to a 1 h cis-DDP pulse revealed that synergism occurred only when the two drugs were present simultaneously. The inactivating effect of 10 μM cis-DDP in combination with 2 mM NY 3170 given to synchronized NHIK 3025 cells at various stages of the cell cycle was also determined. For cells treated in S or in G2+M cell survival was reduced by a factor of 5 after a 1 h treatment with the drug combination as compared to similar treatment with cis-DDP alone. The cells appeared to be most sensitive at the time of initiation of DNA synthesis. Here cell survival was reduced by a factor of 100 following treatment with the drug combination than following treatment with cis-DDP alone. Measurement of cell-associated platinum by atomic absorption spectroscopy indicated that cellular uptake of cis-DDP was increased when NY 3170 was simultaneously present during drug treatment.

Materials and methods

Cell line and cell synchronization

NHIK 3025 cells originating from a human cervical carcinoma in situ (Nordbye & Oftebro, 1969; Oftebro & Nordbye, 1969) were cultivated as monolayers in medium E2a (Puck et al., 1957) supplemented with 20% human and 10% horse serum (Gibco Europe Ltd., Renfrewshire, Scotland, UK). Under growth conditions used, the mean cell-cycle phase durations are 6.5 h for G1, 8 h for S and 2.5 h for G2. Mitosis lasts for 1 h. The median cell-cycle time is 18 h (Pettersen et al., 1977). Cells were synchronized by mitotic selection (Pettersen et al., 1977) and seeded immediately into 25 cm² plastic flasks (Falcon 3013, Falcon Plastics, Oxnard, CA, USA). All experiments involving synchronized cells were performed in a 37°C incubator room. The entrance of cells into the various cell-cycle phases was determined by flow cytometry of mithramycin-stained cells as described by Lindmo and Pettersen (1979) and Pettersen et al. (1983). Cell division was recorded in a separate 25 cm² flask where fields of ~100 cells were followed microscopically. The time of each cell division was recorded.

Cell survival

The inactivating effect of drugs was determined using asynchronous NHIK 3025 cells seeded onto 60 x 15 mm style plastic petri dishes (Falcon 3002). A known number of cells was seeded in each of 5 replicate dishes and allowed to attach for 2 h. Drug-containing medium was then added and incubation continued for 1 h. Finally the drug-containing medium was removed, the dishes were rinsed with warm (37°C) Hank’s balanced salt solution and fresh medium was added. Incubation of cells for colony formation was for 12–14 days in a 5% CO₂ incubator holding 37°C. Medium was changed 7 days after the experiment was initiated. Colonies of cells were fixed in ethanol and stained with methylene blue. Colonies consisting of over 40 cells were counted.

For experiments involving synchronized cells, each data point represents counts from 4 replicate flasks. Cells were seeded into flasks immediately after mitotic selection and allowed to attach for 2 h before 1 h drug combination.

1-Propargyl-5-chloropyrimidin-2-one (NY 3170) is a mitotic inhibitor which causes reversible metaphase inhibition in cultured cells (Wibe & Oftebro, 1979). A sufficient, but necessary prerequisite for metaphase inhibition is that the drug be present during mitosis (Wibe et al., 1979). The parent compound 5-chloropyrimidin-2-one (NY 3000) also induces metaphase arrest in cultured cells (Wibe et al., 1978). Both NY 3170 and NY 3000 are members of a new class of mitotic inhibitors, called metahalones, which are based upon a pyrimidine structure (Gacek et al., 1978). NY 3170 binds weakly to tubulin, the major protein of microtubules and the spindle apparatus (Dornish et al., 1984). and is a competitive inhibitor of colchicine binding to tubulin (Dornish & Oftebro, 1983; Dornish et al., 1984a). The combination of NY 3170 and vincristine has shown more than additive effects with respect to cell inactivation when human cells of the cell line NHIK 3025 were treated with both agents (Wibe, 1980).

Cis-Dichlorodiammineplatinum(II) (cis-DDP) is known to act synergistically when administered in combination with various chemotherapeutic drugs (Gale et al., 1977; Schabel et al., 1979). Many pyrimidine compounds in combination with cis-DDP have demonstrated increased cell inactivating effects (Davidson et al., 1975). Pyrimidine bases and nucleosides (Drewinko et al., 1980), 1-beta-D-arabinofuranosycytosine (Bergerat et al., 1981; Drewinko et al., 1980; Schabel et al., 1979) and 5-aza-2'-deoxycytidine (Vesely, 1982) in combination with cis-DDP have displayed marked synergistic effects both in vivo and in vitro. The exact mechanism responsible for synergism between pyrimidine compounds and cis-DDP is not known although Drewinko et al. (1980) have found an increase in the number of DNA cross-links following cis-DDP + 1-beta-D-arabinofuranosycytosine combination treatment.

This report presents results in which NY 3170 (a pyrimidine and a mitotic inhibitor) in combination with cis-DDP (a DNA damaging agent [Zwelling & Kohn, 1979; Zwelling & Kohn, 1980]) displays synergistic cell inactivation of human cells treated in culture.
treatments were initiated at various times after mitotic selection. Cells were then washed with warm (37°C) PBS (NaCl 8,000 mg l⁻¹; Na₂HPO₄:2H₂O 1,150 mg l⁻¹; KH₂PO₄ 200 mg l⁻¹; KCl 200 mg l⁻¹) and fresh medium added. Incubation for colony formation was for 10–12 days at 37°C. Flasks were flushed with 5% CO₂ in air whenever opened. Colonies were fixed and stained as above.

**Atomic absorption spectroscopy**

Analysis of cell-associated platinum was performed using a Varian SpectrAA-30 atomic absorption spectrometer fitted with a GTA-96 graphite tube atomizer. Instrument control and data acquisition was by Varian Atomic Absorption Software. Automatic background correction with a modulated deuterium lamp was utilized. Cells were loosened from flasks by trypsin treatment and counted. A volume equivalent to 2 × 10⁶ cells was pipetted into conical centrifuge tubes, three replicate tubes for each drug concentration. The tubes were centrifuged and cells were resuspended in drug-containing medium, usually 3 ml/tube. The cells were incubated with drugs at 37°C and held in suspension by using a rotary rack. After treatment the cells were centrifuged and washed in phosphate-buffered saline. The cell pellet was taken up into 100 µl concentrated HNO₃. Following overnight oxidation of organic material, 100 µl H₂O was then added to each tube. Aliquots of 25 µl (representing 250,000 cells) were placed in a graphite tube and the atomic absorption signal measured at 265.9 nm following atomization of the sample was registered. Platinum content was quantitated by running a calibration curve immediately before the samples.

**Drugs**

Cis-dichlorodiammineplatinum(II) was purchased from Sigma Chemical Co., St. Louis, MO, USA. 5-Chloropyrimidin-2-one and 1-propargyl-5-chloropyrimidin-2-one (Figure 1) were provided by Prof. S. Laland, Department of Biochemistry, University of Oslo, Oslo, Norway. Drug solutions were made in phosphate-buffered saline or Hanks’ balanced salt solution and sterile filtered. Drug combinations were mixed immediately before use using stock solutions diluted in medium E2a.

![Figure 1 Structure of 5-chloropyrimidin-2-one (NY 3000) and 1-propargyl-5-chloropyrimidin-2-one (NY 3170).](image)

**Results**

Figure 2 shows the surviving fraction of asynchronous (exponentially growing) NHIK 3025 cells following a 1 h treatment with cis-DDP or cis-DDP in combination with 2 mM NY 3170 as a function of the concentration of cis-DDP. NY 3170 alone has little or no effect on the survival of exponentially growing cells after such a short treatment period (Wibe & Oftebro, 1979). The data presented in Figure 2 indicate that the simultaneous presence of 2 mM NY 3170 resulted in an additional inactivating effect corresponding to that which a doubling of the cis-DDP concentration would give.

To study how the increase in cell inactivation by cis-DDP depended upon the concentration of NY 3170, we performed another set of experiments where exponentially growing cells were treated with a combination of a fixed concentration of cis-DDP (either 6 or 10 µM) and NY 3170 at various concentrations. The results are presented in Figure 3 together with data showing the surviving fraction following treatment with NY 3170 alone. As demonstrated, cell inactivation induced by cis-DDP alone was significantly increased by the simultaneous presence of NY 3170.

As NY 3170 is synthesized from NY 3000 (Gacek et al., 1979), the cell inactivating effect of a simultaneous combination of 6 or 10 µM cis-DDP and various concentrations of NY 3000 was also tested. The results in Figure 4 clearly show that the simultaneous presence of up to 5 mM NY 3000 did not have any effect on cis-DDP-induced cell inactivation. These results indicate the apparent importance of the 1-propargyl side group present in NY 3170 for cis-DDP + NY 3170 synergism.

The synergistic effect of NY 3170 and cis-DDP as a function of the duration of drug exposure is presented in Figure 5. In these experiments, NHIK 3025 cells were exposed to 10 µM cis-DDP either alone or in combination with 2 mM NY 3170 for up to 4 h. While treatment with 2 mM NY 3170 alone resulted in very small decreases in cell survival, treatment with 10 µM cis-DDP alone resulted in a survival which decreased with increasing drug exposure times. The simultaneous presence of 10 µM cis-DDP and 2 mM NY 3170 resulted in a large synergistic effect as compared with that after cis-DDP alone. From the data, 1 h treatment with 10 µM cis-DDP + 2 mM NY 3170 resulted in the same survival as 2 h treatment with 10 µM cis-DDP alone.
SYNERGISTIC EFFECT OF cis-DDP AND NY 3170

Figure 3 Surviving fraction of asynchronous NHIK 3025 cells as a function of the concentration of NY 3170 (○), NY 3170+6 μM cis-DDP (▲), or NY 3170+10 μM cis-DDP (△). (■, □) represent 6 μM or 10 μM cis-DDP alone respectively. Experimental conditions and s.e. as in Figure 2.

Figure 4 Surviving fraction of asynchronous NHIK 3025 cells as a function of the concentration of NY 3000 (○), NY 3000+6 μM cis-DDP (▲), or NY 3000+10 μM cis-DDP (△). (■, □) represent 6 μM or 10 μM cis-DDP alone respectively. Experimental conditions and s.e. as in Figure 2.

In the experiments described thus far cis-DDP and NY 3170 were added and removed simultaneously for combined treatment. We have also studied the combined effects when the treatment period for the two drugs were separated or overlapped only partially. The data in Figure 6 represents such an experiment where 10 μM cis-DDP was present for 1 h (horizontal line marking from 0 to 1 h) and NY 3170 (2 mM) was present as 1 h pulses either before, during, or after the cis-DDP treatment period. Survival is plotted as a function of the time when NY 3170 was added. Cells treated with 10 μM cis-DDP alone (squares) or with 2 mM NY 3170 alone (circles) are also shown.

Figure 5 Surviving fraction of asynchronous NHIK 3025 cells as a function of the duration of drug treatment with 2 mM NY 3170 (○), 10 μM cis-DDP (□), and 2 mM NY 3170+10 μM cis-DDP (△). Single cells attached to plastic dishes were treated with each drug or drug combination for the time indicated in the figure. Drug treatment was terminated by removal of the drug-containing medium, thereafter cells were washed in Hanks' balanced salt solution and reincubated with fresh medium for colony formation. Data from 5 replicate dishes were averaged for each experimental point and s.e. is represented by vertical bars.

From Figure 6, NY 3170 (2 mM) given simultaneously with 10 μM cis-DDP resulted in a cell survival reduced to about one-tenth of that following treatment with cis-DDP alone. When NY 3170 was added 0.5 h later than cis-DDP and thus overlapped the pulse of cis-DDP by only 0.5 h, cell survival was now reduced by only one-half of that after cis-DDP alone. NY 3170 treatment immediately following the cis-DDP pulse resulted in identical survival as did treatment with cis-DDP alone. Further separation of NY 3170 treatment after cis-DDP resulted in no further change in survival.

Figure 6 also shows that the same is true when NY 3170 treatment precedes the cis-DDP pulse. Identical survival was measured until the treatment period for the two drugs overlapped by 0.5 h. When NY 3170 treatment preceded that of cis-DDP by 0.5 h, cell survival was again about one-half that following cis-DDP treatment alone. Maximum synergism with respect to cell inactivation thus occurs only when the two drugs are present simultaneously.

To determine whether the increase in cis-DDP-induced cell inactivation by NY 3170 was specific to any particular phase of the cell cycle, we treated synchronized cell populations with 1 h pulses of 10 μM cis-DDP alone or in combination with 2 mM NY 3170 at various times after mitotic selection. The results are shown in Figure 7. The synergistic effect of NY 3170 in combination with cis-DDP was present throughout the cell cycle. Cells in late G1/early S appeared to be the most sensitive where survival following treatment with cis-DDP+NY 3170 was reduced by a factor of 100 compared to that following cis-DDP treatment alone.
Furthermore, a 1 h drug combination treatment in S and G2 resulted in a reduction of cell survival to about one-fifth of that following cis-DDP treatment alone.

Since the cell survival experiments indicated that treatment of cells with cis-DDP in combination with NY 3170 mimic a doubling of cis-DDP concentration, it was of interest to quantitate the platinum content in treated cells. Measurements of cell-associated platinum as performed by use of flameless atomic absorption spectroscopy are shown in Figure 8. The amount of cell-associated platinum in asynchronous cells treated for 1 h (open squares) or 2 h (filled squares) with cis-DDP increased as the concentration of cis-DDP in cell culture medium increased. The simultaneous presence of 2 mM NY 3170, however, caused an apparent doubling of the amount of cell-associated platinum. After a 1 h treatment with cis-DDP in combination with NY 3170 the amount of cell-associated platinum was the same as after a 2 h treatment with cis-DDP alone.

**Figure 6** Surviving fraction of asynchronous NIH3T3 3025 cells treated with 10 μM cis-DDP as a function of scheduling of 1 h pulses of 2 mM NY 3170 (Δ), (C) and (□) represent cell survival after a 1 h pulse of 2 mM NY 3170 alone or 10 μM cis-DDP alone respectively. The horizontal bar represents the treatment period for cis-DDP and the data points are plotted at the time at which drug incubation began. Each experimental point represents the mean of 5 replicate dishes. Vertical bars represent s.e.

**Figure 7** Surviving fraction of synchronized NIH3T3 3025 cells treated for 1 h with 2 mM NY 3170 (○), 10 μM cis-DDP (□), or 2 mM NY 3170 + 10 μM cis-DDP (△) as a function of the time after mitotic selection. Experimental points from 1 typical experiment are plotted at the time at which drug incubation was begun. The duration of the various cell-cycle phases for control cells is indicated at the bottom of the figure. Each experimental point represents the mean colony count from 4 replicate flasks. The vertical bars represent s.e.

**Figure 8** The platinum content in NIH3T3 3025 cells measured by flameless atomic absorption spectroscopy as a function of the concentration of cis-DDP in cell culture medium. Cells were treated for 1 h (open symbols) or 2 h (filled symbols) with various concentrations of cis-DDP alone (○, □) or in combination with 2 mM NY 3170 (△, ▲). The cells were then washed in PBS and organic material in the cell pellet was oxidized with HNO₃. Platinum content in an aliquot representing 250,000 cells was quantitated in triplicate for each drug concentration using the 265.9 nm absorbance line of platinum. Background correction utilizing a modulated deuterium lamp was applied. Vertical bars represent s.e.

**Discussion**

The mitotic inhibitor 1-propargyl-5-chloropirimidin-2-one previously shown to exert a synergistic effect in combination with vincristine (Wibe, 1980), also displayed synergism with respect to cell inactivation when NIH3T3 3025 human cells were treated with the drug in combination with cis-DDP (Figures 2, 3 and 5). Analysis of the data in Figure 2 reveals that the synergistic effect of NY 3170 is not of a dose-modifying type, but that it increases with increasing concentrations of cis-DDP. This is seen from Figure 2 by calculating the ratio between the cis-DDP concentrations in the absence and presence of NY 3170 at various survival levels. While this ratio is 1.6 at the survival level of 0.5, it is 2.0 at the survival level of 0.001. Thus, NY 3170 does not just increase the biological effects of cis-DDP by a certain factor, but its effect depends upon the cis-DDP concentration.

The maximal cis-DDP + NY 3170 synergism was found for 2 mM NY 3170 (Figure 3) even though this concentration of NY 3170 had no effect on cell inactivation alone. Higher concentrations of NY 3170 alone did, however, inactivate cells, thus 2 mM was the limit of this investigation. With respect to the duration of drug exposure, the presence of 2 mM NY 3170 together with cis-DDP caused similar cell survivals as would a doubling of the time of treatment with cis-DDP alone (Figure 5). Thus NY 3170 in combination
with cis-DDP appears to cause cell inactivating effects that mimic either a doubling of cis-DDP concentration or treatment times.

The parent compound, 5-chloropirimidin-2-one, also possessing metaphase inhibitory properties (Gacek et al., 1979; Wibe et al., 1978) did not display any effect on cis-DDP-induced cell inactivation (Figure 4). The only structural differences between these two molecules is the 1-propargyl group present in NY 3170 (see Figure 1).

Results from scheduling NY 3170 administration (Figure 6) show that NY 3170 must be present simultaneously with cis-DDP in order for the drug combination to exert a synergistic cell inactivating effect. When NY 3170 treatment either preceded or followed that of cis-DDP, cell survival was identical to that following treatment with cis-DDP alone. Sligt overlapping of the two treatments resulted in a reduction in cell survival by about one-half for the drug combination than for cis-DDP alone. Simultaneous drug treatment resulted in a reduction of cell survival to about one-tenth of that following a 1 h pulse with cis-DDP alone.

From the above results two alternative assumptions are possible: (1) cis-DDP and NY 3170 could react to form a third compound of greater toxicity. (2) NY 3170 could increase cellular uptake of cis-DDP.

Regarding the first assumption we know that NY 3170 is able to penetrate cells quite readily (Wibe & Oftebro, 1981). Intracellular cis-DDP forms an aquted platinum species which react with nucleophilic sites (Zwelling & Kohn, 1979). The carbon-carbon triple bond of the propargyl group of NY 3170 may act as such a site. We have observed a reaction between cis-DDP and NY 3170 (data not shown), however, the kinetics of this reaction are too slow to explain the observed synergism between the two drugs. Nevertheless, we cannot rule out the possibility that just such a toxic reaction may be responsible for the large increase in cell inactivation seen when synchronized cells are treated in G1-phase.

From Figure 7 one can see that treatment of synchronized cells with cis-DDP alone caused an increased inactivation of those cells in early G1-phase. Combination treatment of cis-DDP together with NY 3170, however, caused a very large inactivation of cells in late G1-phase (i.e. from 6 to 8h after mitotic selection) even though the two drugs were present for only 1h. Perhaps this difference in cell-cycle specific inactivation may be due to just such a reaction between cis-DDP and NY 3170. We know that initiation of DNA synthesis in NIHK 3025 cells takes place from about 4 to 8h after the end of mitosis (unpublished results). Although an unproven hypothesis, a possible reaction product between cis-DDP and NY 3170 albeir in small quantities, could block the initiation of DNA synthesis and cause irreversible damage to cells in which DNA synthesis was already initiated.

With respect to the second assumption we have previously shown that benzaldehyde and the vitamin B$_6$ aldehydes pyridoxal and pyridoxal 5-phosphate modulate cellular toxicity of cis-DDP (Dornish et al., 1984b, 1986; Dornish & Pettersen, 1985). In contrast to NY 3170, these aldehydes induced a protective effect in combination with cis-DDP. As it turned out, the protective effect was a result of reduced cellular uptake of cis-DDP due to the aldehydes. It is not known whether it is passive diffusion of cis-DDP (Gale et al., 1973) or membrane-mediated transport (Byfield & Calabro-Jones, 1981), or both, that is influenced by the aldehydes. One can not exclude, however, that NY 3170 could leave the opposite effect and increase cellular uptake of cis-DDP due to a membrane effect leading to enhanced cytotoxicity. Increased uptake of cis-DDP would also explain why NY 3170 potentiates the cell inactivating effect of cis-DDP in all stages of the cell cycle (Figure 7).

The results presented in Figure 8 confirm that, indeed, NY 3170 induces an increased uptake of cis-DDP into NIHK 3025 cells. Increased uptake of cis-DDP by NY 3170 is transient as shown by scheduling experiments in Figure 6 where the drugs must be in simultaneous combination. This could certainly be a membrane-mediated response to NY 3170. Finally, the structural moiety responsible for the synergistic effect must reside within the propargyl side group of NY 3170 since no such increase in cis-DDP uptake was seen when cells were treated with NY 3000 and cis-DDP (data not shown).

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