Interactions of doxorubicin and cis-platin in squamous carcinoma cells in culture

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Summary

Doxorubicin (DXR) has a positive inoculum effect and penetrates poorly into the core of multicellular tumour spheroids (MTS). Cis-platin (DDP) displays neither of these characteristics. We evaluated whether combining these 2 agents would influence the cell kill effect at a tumour mass level. MTS were produced from a PC-10 squamous lung carcinoma cell line. MTS were exposed to either drug first for 1 h with different intervals between exposure. Cells were then trypsinized to a single cell suspension and subjected to clonogenic assay. Combination effects were analyzed by median effect plot analysis. The more MTS ml⁻¹ medium, the lower the cell kill effect of DXR. Simultaneous exposure to the 2 drugs was synergistic. DXR exposure first followed by DDP was less efficacious than, or the same as, the simultaneous exposure. In contrast, DDP followed by DXR was more efficacious with the best cell kill at a 1 h interval between each drug. This phenomenon was observed even at non-toxic doses of DDP. The fluorescent microscopic study of DXR indicated that prior exposure of MTS to DDP resulted in increased DXR penetration into the MTS core leading to heightened synergism with this sequence. These data suggest that the proper combination of DXR plus DDP should be in sequence with DDP first. Clinical, toxicological and pharmacological trials of DDP administration first, followed by DXR, are warranted.

Materials and methods

Human tumour cell line

PC-10 squamous lung carcinoma cell line was used in these experiments (Kinjo et al., 1979). Cells were maintained as a monolayer in RPMI-1640 medium (GIBCO, Grand Island, NY), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Gibco) at 37°C in a 5% CO₂/95% humidified air atmosphere. These cells were subcultured after trypsinization with 0.2% trypsin (Type III from bovine pancreas, Sigma, St Louis, MD) and 0.01% EDTA in Hank’s balanced salt solution (HBSS) (Gibco).

Growth of MTS

MTS were developed by a liquid overlay culture technique (Yuhas et al., 1977), as described previously (Kohno et al., 1987). Aliquots of 1 x 10⁴ cells in 10 ml of complete culture medium were placed in 100 mm plastic Petri dishes (Falcon 1005, Cockeysville, MD) previously coated with 0.5% agar (Noble, Difco, Detroit, MI) in the same culture medium. These cells were incubated in 5% CO₂/95% humidified air at 37°C. When MTS were formed, they were transferred to a new agar dish once a week.

Drugs

DXR was purchased from Adria Laboratories, Columbus, OH and DDP was purchased from Bristol Laboratories, Syracuse, NY.

Conditions of drug exposure and determination of cell survival

After 3 weeks of culture, MTS with a diameter of ~700 μm were formed. MTS with a diameter >700 μm tended to develop a necrotic core. MTS with ~700 μm were transferred into a new agar-coated multiwell plate (Falcon 3046, Becton Dickinson Labware, Lincoln Park, NJ) containing 2 ml fresh medium. After a preincubation period of 24 h, MTS in different densities were exposed to graded concentrations of DXR or DDP for 1 h. The MTS were then gently washed twice with PBS (Gibco). The single cell suspensions were made by exposure to the 0.2% trypsin and 0.01% EDTA solution for 10 min at 37°C, followed by mechanical disaggregation through repeated pipetting. These cells were washed once with the medium and resuspended in the medium. One tenth ml aliquots of cell suspension (3,000 cells) were seeded on 60 mm Petri dishes (Corning 25011, Corning, NY) containing 0.5% noble agar in complete culture medium for clonogenic assay (Kuroki, 1974). The dishes were incubated for 10 days at 37°C, under 5% CO₂/95% humidified air. Colonies of ≥50 cells were counted. The plating efficiency of untreated cells under these conditions was ~40%. The dose response curve was drawn by plotting the number of colonies as a percentage of control against each drug concentration. Each experiment was done in triplicate and repeated at least 3 times.

Combination experiments were carried out as a 1 h exposure to each drug. After drug exposure, cells were washed free of drug and incubated in drug-free culture medium for different intervals. After the indicated incubation time periods, the MTS were exposed to a second drug for 1 h, washed free of drug, trypsinized to a single cell suspension and subjected to clonogenic assay.

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Data analysis

The efficacy of the combination was determined by the median effect plot analysis using an IBM PC microcomputer system (Chou & Talalay, 1984, 1987; Chou, 1985). This method involves plotting dose-effect curves for each drug for one or more multiple-dilutions and fixed ratio combinations of the drugs using the median effect equation: \( fa/fu = (D/D_m)^m \), where \( D \) is the dose, \( D_m \) is the dose required for 50% effect (e.g. 50% inhibition of PC-10 cell's colony formation), \( fa \) is the fraction affected by the dose \( D \), \( fu \) the fraction unaffected and \( m \) a coefficient signifying the sigmoidicity of the dose-effect curve. The dose-effect curve was plotted using a logarithmic conversion of this equation which determines the \( m \) and \( D_m \) values. Based on the slope of the dose-effect curves, it can be decided whether the agents have mutually exclusive effects (e.g. similar mode of action) or mutually non-exclusive effects (e.g. independent mode of action). A combination index (CI) was then determined using the equation:

\[
CI = \frac{(Dx_1)}{(Dx_2)} = \frac{(D_1)}{(D_2)} \times \frac{(D_2)}{(D_1)} = \frac{(D_1)}{(D_2)} \times \frac{(D_2)}{(D_1)}
\]

where \((Dx_1)\) and \((Dx_2)\) are the doses of drugs 1 and 2, respectively, required to produce \( x\% \) effect individually. \((Dx_1)\) and \((Dx_2)\) for \( x\% \) cell kill can be determined by drawing a least square regression line on the computer graphic system. \((D_1)\) and \((D_2)\) are the doses of drugs 1 and 2, respectively, which are required to produce the same \( x\% \) effect in combination. If the drugs are mutually exclusive, then \( x = 0 \); if mutually non-exclusive, then \( x = 1 \). When \( CI = 1 \), the interaction was considered additive; when \( CI > 1 \), synergism was indicated; and when \( CI > 1 \), antagonism was indicated.

Fluorescent microscopy

MTS of \( \sim 700 \mu \text{m} \) diam. were treated with \( 2 \times 10^{-4} \text{M} \) of DXR alone or combination of DXR and \( 2 \times 10^{-3} \text{M} \) DDP at 1h intervals of each drug, which was the best cell kill sequence. The treated MTS were washed with ice-cold PBS once, embedded in OCT compound (Miles Scientific, IL) and frozen rapidly. Thin sections (5 \mu m) were made using a cryotome (Lipshaw Elect, MI). DXR fluorescence was observed under a fluorescent microscope equipped with epilumination (Nikon, DS-EPI-FL, Japan) using G-Green (excitation filter 535-580 \mu m, barrier filter 580 \mu m). In this system endogenous fluorescence from control MTS (ones not exposed to DXR) could not be recognized.

Results

The influence of MTS density on DXR- or DDP-induced cell lethality is shown in Figure 1. For DXR-induced cell lethality, the higher the density of MTS, the lesser the cell kill effects and dose-response curves of DXR progressively flattened at high drug concentrations. In contrast, DDP gave entirely different dose-effect curves; cell survival curves for high MTS density and low density overlapped each other and there was progressively increasing cell kill at increasing doses.

Time dependent cell lethality of the combination of DXR plus DDP at low MTS density is illustrated in Figure 2. Three lines are shown for 3 different concentrations of the drug. DXR exposure followed by DDP was less efficacious than, or the same as, simultaneous exposure. In contrast, exposure to DDP followed by DXR was more efficacious; the best cell kill with a 1h interval between each drug. Increasing time intervals from DDP to DXR for longer than 1h resulted in a gradual diminution of the potentiation.

The influence of MTS density on time-dependent cell lethality of DXR plus DDP combination is shown in Figure 3. The differences of the 2 lines on the left half of the panel indicate weak activity of the combination at high MTS density when DXR is administered first. When the 2 drugs were given simultaneously, the survival fractions were nearly identical, indicative of better expression of DDP activity. For both low and high MTS densities, the best cell kill sequence of this combination was DDP followed by DXR with a 1h interval between the 2 drugs. When the interval between DDP followed by DXR widened, the 2 lines began to separate again, suggesting the same effect of MTS density seen when DXR was given first.

Examples of the median effect plot analysis from the combination of DDP followed by DXR for 24h later by DDP, simultaneous exposure of the 2 drugs and DDP followed 1h later by DXR – are shown in Figure 4. All of these 3 lines showed synergistic interactions; among them, the DDP expo-
These non-toxic concentrations of cis-platin on doxorubicin-induced cell lethality. Data are shown as survival fraction in percentage compared to doxorubicin alone control (± s.d.)

| Cis-platin (M) | Monolayer | Multicellular tumour spheroids |
|---------------|-----------|-------------------------------|
| 0            | 100.0a    | 100.0b                        |
| 1 × 10^{-9} | 103.4±0.9  | ND                            |
| 1 × 10^{-8} | 103.4±1.5  | 86.0±7.8                      |
| 1 × 10^{-7} | 92.9±6.5   | 71.7±3.8                      |
| 1 × 10^{-6} | 84.9±7.7   | 63.7±9.6                      |

*1 × 10^{-6}M doxorubicin alone; *2 × 10^{-3}M doxorubicin alone. *The asterisks indicate significant (P<0.05) decrease in survival fraction as compared to doxorubicin alone control; ND, not done.

Figure 5 Panel a shows doxorubicin fluorescence of spheroid cross-section after 1h exposure to the compound alone. Panel b shows doxorubicin fluorescence of spheroid cross-section after pre-treatment with cis-platin.

Discussion

MTS have certain characteristics similar to de novo solid tumours (Allison et al., 1983; Franko & Sutherland, 1979; Freyer & Sutherland, 1980; Nederman et al., 1984; Sutherland & Durant, 1973; Yuhas et al., 1977, 1978). They contain such extracellular matrix as fibronectin, laminin and collagen (Nederman & Twentyman, 1984), comprise a chronically hypoxic cell population in the core (Franko & Sutherland, 1979; Sutherland & Durant, 1973) and show heterogeneous cell cycle times (Allison et al., 1983; Freyer & Sutherland, 1980). For these reasons, MTS have been used as an in vitro model to study the effects of radiation (Allison et al., 1983; Franko & Sutherland, 1979; Freyer & Sutherland, 1980; Sasaki et al., 1984a; Sutherland & Durant, 1973) and chemotherapeutic agents (Erlichman & Vidgen, 1984; Kerr et al., 1986; Nederman & Twentyman, 1984; Sasaki et al., 1984b; West et al., 1980).

Our data show that the density of MTS influences the cell kill effect of certain drugs. The higher the density of MTS, the lesser the cell kill effect of DXR. The concept of inoculum effect seen at a single cell level could thus be extended to the density of MTS. Since solid tumour masses in vitro do not grow more than 300-400 μm in diameter without a blood supply (Folkman, 1986; Kolstad, 1968), we
have assumed that a large clinically recognizable tumour mass with neovascularure is equivalent to MTS in high density. The DDP-induced cell kill effect seemed to follow first order kinetics irrespective of MTS density, indicating good drug penetration into the MTS core. In contrast, the cell kill effect of DXR was progressively less efficacious at higher drug concentrations as a consequence of poor drug penetration.

The combination study herein presented shows that sequencing DDP and DXR influenced the cell kill effect at a tumour mass level. As shown in Figure 4, DDP plus DXR was always synergistic, with the exception of very low concentrations of the drugs. With increasing effect levels the combination index was progressively lower indicating heightened synergism. The drug sequencing studies showed that the best cell kill sequence was DDP first with a 1 h interval before DXR exposure. The increased synergistic interaction of this sequence was shown to be due to an increased population of cells in MTS at risk.

In attempts to elucidate the mechanism of the heightened cell kill effect from this sequence, we evaluated whether DDP could influence cell lethality and penetration of DXR. From demonstration of DDP increased DXR-induced cell lethality even at non-toxic low concentration levels for MTS. This was not observed for monolayers. For fluorescent microscopy study, DXR concentrations of \( \geq 2 \times 10^{-5} \text{M} \) were necessary in order to detect the fluorescence. Exposure to DXR alone resulted in DXR fluorescence only on one or two outer layer(s) of the MTS (Inoue et al., 1985). When MTS were exposed to DDP and DXR in this sequence, DXR fluorescence was seen throughout the MTS, indicating good penetration of DXR. The precise mechanism of DDP-induced improvement in DXR penetration is unclear. Other workers indicated that drug penetration into MTS is influenced by (a) cell to cell interaction in MTS, (b) molecular size of the drug, (c) liquid solubility of the drug and (d) intracellular pH gradients (Elrichman & Vidgen, 1984; Kerr et al., 1986). It is likely that DDP exposure changed cell-to-cell interactions within the MTS, making it easier for DXR to penetrate into the core. While the interaction between DDP and cell membrane is poorly understood, it has been observed that the compound binds with cell surface DNA, leading to the loss of the nucleic acid (Juckett & Rosenberg, 1982). Elrichman and Vidgen (1984) reported that avid binding of DXR to the outer layer of MTS inhibited penetration of the drug into the core. It is possible that the avid binding of DXR to cell surface material such as DNA was inhibited by pretreatment with DDP.

Theoretical and practical aspects of combination chemotherapy at a single cell level have been discussed by Sartorelli and Creasey (1982). Little information has so far been provided, however, on the approach with combination chemotherapy at the tumour mass level. Sensitivity of a cell to DRR and DDP is known to be highly related to the intracellular drug concentration (Elrichzolt-Wirth & Hietel, 1986; Iliakis & Lazar, 1987). Any means to increase the drug penetration into MTS should improve drug concentration within the cell.

Our observations indicate that initial treatment with DDP resulted in increased efficacy for DXR at the tumour mass level. Clinical, toxicological and pharmacological trials of DDP followed by DXR are warranted.

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