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Relationship between tumor cell density and drug concentration and the cytotoxic effects of doxorubicin or vincristine: mechanism of inoculum effects*

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Summary. When tumor cell density increases, the cytotoxic activity of certain anticancer agents, such as vincristine (VCR) and doxorubicin (DXR), progressively decreases. This phenomenon is termed the inoculum effect. Since VCR and DXR are less active in an acidic environment, we questioned whether the inoculum effects could have resulted from acidification of the medium that may have developed due to the high cell density. However, measurements of the cytotoxic activity of these agents in a pH-controlled medium revealed only a minor correction of the inoculum effects. Second, we wondered whether the inoculum effects that occurred at the high cell density might have been attributable to insufficient amounts of drugs to bind all the binding sites of the cells. To test this hypothesis, we used drug-resistant sublines, which required higher VCR or DXR concentrations for cell killing than did the parent cell line. When higher drug concentrations were used, the dose-response curves generated for low- and high-density cell populations became closer and overlapped each other, resulting in virtual disappearance of the inoculum effects. Measurements of cellular drug levels revealed that at a high cell density, cells accumulated much smaller amounts of both VCR and DXR in parallel with the positive inoculum effect. In contrast, when high concentrations of the drugs were used in drug-resistant cells, differences in the cellular drug contents between low and high cell densities became narrow. Cisplatin (DDP) belongs to a group of drugs that do not produce inoculum effects, and DDP's cytotoxic effects were not influenced by the pH-controlled medium or by the use of drug-resistant cell lines. These observations indicate that the inoculum effects are the result of the unavailability of VCR or DXR molecules to all cellular binding sites when cells at high densities are exposed to drugs. The drug concentration relative to cell density was apparently the major determinant for the inoculum effects seen in VCR- or DXR-induced cell killing.

Introduction

The cytotoxic effects of certain antineoplastic agents such as vincristine (VCR), doxorubicin (DXR), and bleomycin become progressively less efficacious when cell density is increased. This phenomenon is termed the inoculum effect [11, 12]. Inoculum effects were originally demonstrated in microbial systems in which the size of the inhibitory zone produced by certain antibiotics was influenced by the inoculum size of the microorganisms tested [3, 4]. The exact mechanisms underlying the inoculum effects observed for antitumor agents remain unclear. A high cell density might have caused an increased rate of acidification of the medium. The cellular uptake of both VCR and DXR is known to be decreased at low pH, and these drugs are less efficacious in an acidic environment [5, 6, 8, 13]. A previous study using DXR showed that preincubation of the drug with a high density of tumor cells resulted in a decrease in the cell kill, suggesting that DXR was either inactivated or absorbed by the cell [11]. A subsequent study showed that DXR's reduced cytotoxicity at high cell densities correlated with decreased accumulation of the drug [12]. Thus, an alternative hypothesis would be that the inoculum effects produced at the high cell density might have resulted from the insufficient availability of DXR molecules to bind all the binding sites for an optimal cell kill.

To clarify the mechanisms of inoculum effects, in the present study, we investigated both the influence of the environmental pH and the effect of drug concentrations on the inoculum effects. Furthermore, to clarify the relationship between tumor cell density and drug availability, we measured intracellular contents of VCR or DXR after incubation with cell suspensions at various cell dinsities.

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Materials and methods

Cell lines. The MOLT-3 acute lymphoblastic leukemia cell line [9] and drug-resistant sublines were maintained in RPMI-1640 medium (Gibco, Grand Island, N.Y.) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and were fed twice a week with fresh medium. The drug-resistant sublines used included MOLT-3/VCR_{10}, MOLT-3/VCR_{100}, and MOLT-3/$VCR_{1,000}$ (10-, 100-, and 1,000-fold VCR-resistant, respectively) [1] and MOLT-3/TMQ_{200} and MOLT-3/$TMQ_{2,500}$ (200- and 2,500-fold trimetrexate (TMQ)-resistant, respectively) [2]. Cells in exponential growth at >90% viability were used for all experiments. Cells were periodically examined for mycoplasma contamination with the MycoTect kit (Gibco) and were found to be negative.

Drug exposure and determination of inoculum effect. VCR sulfate and DXR hydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). DDP was purchased from Bristol Laboratories (Evansville, Ind.). VCR and DXR were used as representatives of drugs known to produce inoculum effects [11]. DDP belongs to the group of drugs that produce no inoculum effects. Drug powders were initially reconstituted according to the accompanying instructions and were further diluted in Dulbecco's phosphate-buffered saline (PBS, Gibco). The drug dilutions were freshly prepared for each experiment. RPMI-1640 medium containing 10% FBS was used for all experiments. For experiments using pH-controlled medium, the pH of the medium was adjusted to pH 7.2 with 0.35 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) for maintenance of constant pH throughout the 1-h incubation period. A 1-h exposure to the medium containing 0.35 M HEPES did not affect cell viability or subsequent cell growth. As an additional test to determine the influence of pH, the pH of the medium was adjusted to 6.3 with acetic acid for the low cell density of 1×10^6 cells/ml, and dose-response curves were constructed as described below.

The cells were suspended in fresh RPMI-1640 medium containing 10% FBS at a cell density of 1.1×10^6 or 1.1×10^8 viable cells/ml. Aliquots of 0.9 ml cell suspension were placed in tissue-culture tubes (Falcon 3033, Becton-Dickinson, Cockeysville, Md.) and exposed to 0.1-ml graded concentrations of DXR, VCR, or DDP for 1 h at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air. For control, 0.1 ml PBS was added to the cell suspension instead of drug solution. Addition of the drug solution at the concentration ranges studied did not alter the pH. During exposure, cell suspensions were shaken at 15-min intervals.

At the end of the incubation period, the cells were washed twice with RPMI-1640 medium containing 10% FBS, and the cell density was readjusted to 5×10^4 cells/ml for MOLT-3 parent cells and to 7×10^4 cells/ml for resistant sublines. The reason for using a higher concentration for the resistant sublines is that the doubling times of the resistant sublines are slightly longer than those of the parent cell line; thereby making the absorbance for the untreated control wells is rendered constant for the assay described below. Each 1-ml aliquot of cell suspension was placed in a 24-well flat-bottomed plate (Corning Glass Works, Corning, N.Y.) and then incubated for 3 days at 37° C in a humidified atmosphere comprising 5% CO_2 and 95% air. Following incubation, viable cells were quantitated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma) assay [10, 12].

Briefly, 50 µl MTT solution (4 mg/ml) and 50 µl 0.1 M sodium succinate were added to each well and the plates were further incubated at 37° C for 3 h. Next, the plates were centrifuged at 1,500 g for 10 min at 4° C and the supernatants were removed. The formazan crystals formed were solubilized by the addition of 750 µl dimethylsulfoxide, after which the contents of the wells were thoroughly mixed on a plate shaker for 5 min and the absorbance of each well was measured at 540 nm using a spectrophotometer (Perkin Elmer, Norwalk, Conn.). Dose-response curves were drawn by plotting the absorbance at 540 nm, expressed as a percentage of the untreated control value, against the drug concentrations tested. Preliminary data indicated that the absorbance and the viable cell number showed a linear relationship at cell densities of up to 7×10^5 cells/ml. Each experiment was performed in triplicate and was repeated at least twice.

Measurement of cellular drug levels. Cellular contents of VCR and DXR were measured by a procedure described by Ferguson et al. [6], with a minor modification. Radiolabeled drugs ([^{3}H]-VCR, sp. act. 8.67 mCi/mg; [^{14}C]-DXR, sp. act. 94.5 µCi/mg; Amersham Radiochemicals, Arlington Heights, Ill.) were diluted with respective non-radioactive drugs and were added to the cell suspension at a final cell density of 1×10^6 or 1×10^8 viable cells/ml pH-controlled RPMI-1640 medium plus 10% FBS. After a 1-h period of incubation at 37° C in a shaking incubator, 1-ml aliquots of the reaction mixture were transferred to 1.5-ml polypropylene microcentrifuge tubes (Marsh Biomedical, Rochester, N.Y.) and centrifuged for 1 min in a microcentrifuge. The supernatants were discarded by aspiration, and the cell pellets were washed by resuspension in 1 ml ice-cold PBS followed by further centrifugation. After removal of the supernatant, the cell pellets were solubilized in 1 ml 1% Triton X-100 (v/v) and transferred to counting vials containing scintillant BCS (Amersham). The radioactivity in the cell pellets was determined by a liquid scintillation counter (Packard, Model 1900CA, Downers Grove, IL), and the results were expressed as picomoles of drug per 10^6 cells.

Results

Influence of medium acidification

Figure 1 A shows the sensitivity to VCR of MOLT-3 cells as measured at a cell density of 10^6 or 10^8 cells/ml of the original medium or of the pH-controlled medium. When drug effects were measured in the original medium, as previously reported, an increase in cell density from 10^6 to 10^8 cells/ml resulted in an approximately 10-fold decrease in VCR's cytotoxic effect (positive inoculum effect) as determined by comparison of ID_{50} values (the drug concentration that produces a cytotoxic effect amounting to 50% of control values). At the high cell density, the pH of the medium containing VCR had fallen from 7.2 to 6.3 by the end of the 1-h incubation period, whereas at the low cell density it remained virtually constant at 7.2 throughout the incubation period. Next, the pH of the medium was adjusted to pH 7.2 with 0.35 M HEPES, and cellular sensitivity to VCR was measured. In this experiment, the pH of the medium was maintained at 7.2 throughout the 1-h incubation period. Under this condition, the dose-response curve for the high cell density moved slightly to the left, but the presence of definite inoculum effects was maintained.

To examine whether the phenomenon described above for VCR could be observed using other drugs, we examined DXR in a similar manner (Fig. 1 B). An increase in cell density from 10^6 to 10^8 cells/ml resulted in an approximately 40-fold decrease in DXR's cytotoxic effects. Again, at the high cell density, the pH of the medium fell from 7.2 to 6.3 in 1 h, whereas at the low cell density it remained constant at pH 7.2. When DXR sensitivity was measured in the medium containing 0.35 M HEPES, the dose-response curve for the high-density cells moved to the left; however, the positive inoculum effects were again maintained. Confirmatory experiments carried out in a medium of pH 6.3 again showed decreased cell-kill effects for VCR and DXR as compared with those obtained using the original medium. However, adjustment of pH alone failed to eliminate the inoculum effects (data not shown).

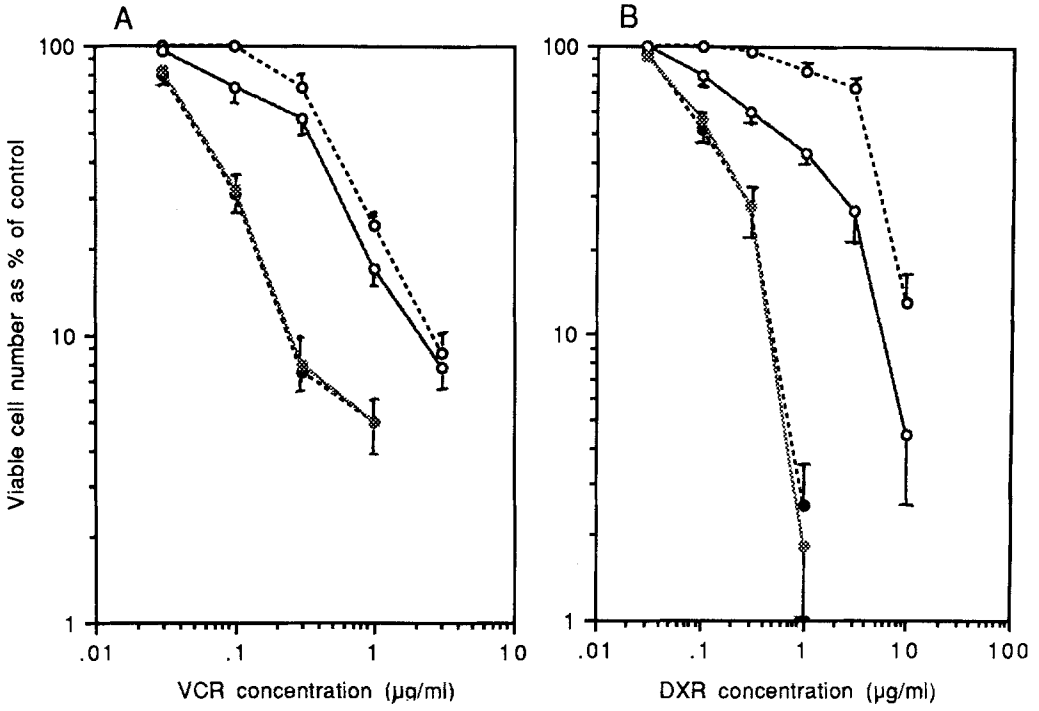

Fig. 1 A, B. The sensitivity to **A** VCR and **B** DXR of MOLT-3 cells at a cell density of 10^6 (●) or 10^8 (○) cells/ml RPMI-1640 medium containing 10% FBS (*broken lines*) and those in pH-controlled RPMI-1640 medium plus 10% FBS (*solid lines*). Each data point and bar represents the mean value ± SD for at least 2 experiments performed in triplicate

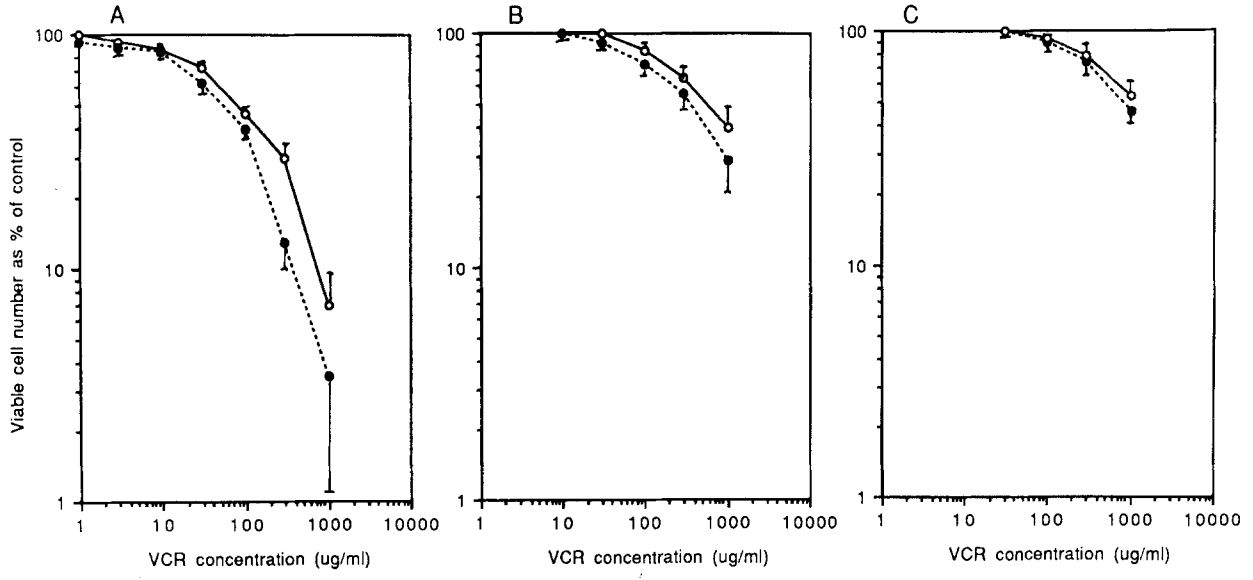

Fig. 2 A–C. The sensitivity to VCR of **A** MOLT-3/VCR_{10} cells, **B** MOLT-3/VCR_{100} cells, and **C** MOLT-3/TMQ_{200} cells at a cell density of 10^6 (●) or 10^8 (○) cells/ml pH-controlled RPMI-1640 medium plus 10% FBS. Each data point and bar represents the mean value ± SD for at least 2 experiments performed in triplicate

Dose-response curves generated for DDP-induced cytotoxic effects in the pH-controlled medium were essentially identical to those obtained using the original medium, irrespective of the cell density (data not shown). Thus, DDP was devoid of inoculum effects under any of the conditions studied.

Influence of drug concentration

The second possibility we investigated was whether the amount of drug molecules available to the receptor or binding sites of all the cells might be insufficient in cell populations of high density, with greater numbers of cells therefore remaining unaffected. To test this possibility, we evaluated VCR's inoculum effects in the pH-controlled medium using MOLT-3/VCR_{10}, MOLT-3/VCR_{100}, and MOLT-3/$TMQ_{2,500}$ cells, whose ID_{50} values were much higher than those of the parent cells. The results are shown in Fig. 2. When larger amounts of drugs were used, the dose-response curves generated for high- and low-density cells became closer to each other, and the inoculum effects virtually disappeared. The results were similar, irrespective of the drug-resistant sublines used, indicating that drug

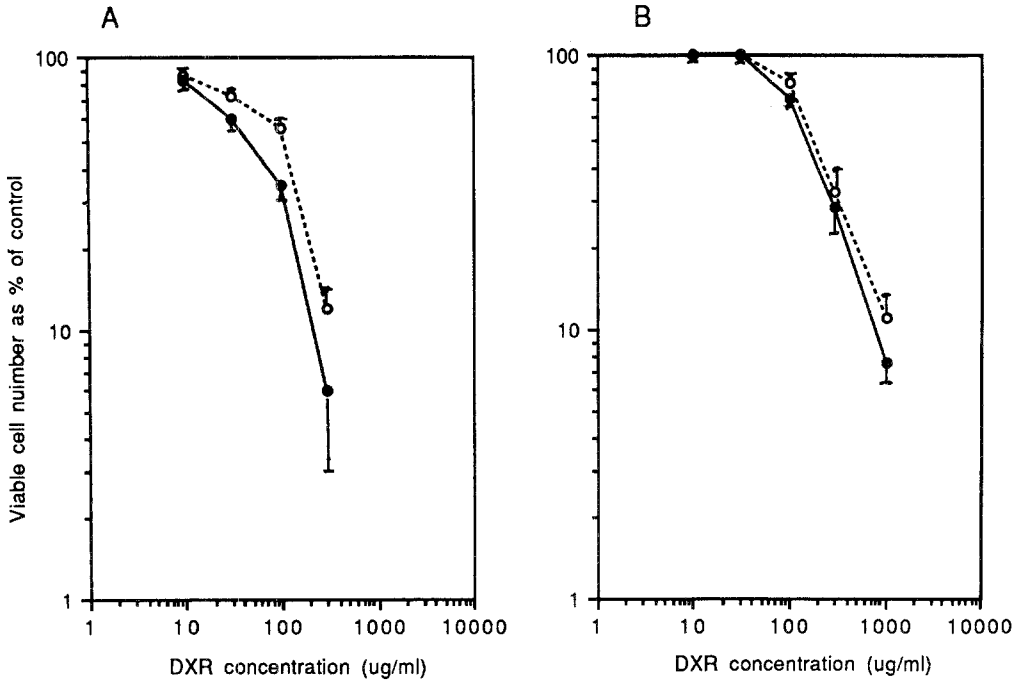

Fig. 3 A, B. The sensitivity to DXR of **A** MOLT-3/$TMQ_{2,500}$ cells and **B** MOLT-3/$VCR_{1,000}$ cells at a cell density of 10^6 (●) or 10^8 (○) cells/ml pH-controlled RPMI-1640 medium plus 10% FBS. Each data point and bar represents the mean value ± SD for at least 2 experiments performed in triplicate

Table 1. Cellular contents of VCR and DXR under various exposure conditions

Cell density (cells/ml)	Cellular content of VCR (pmol/10^6 cells)		Cellular content of DXR (pmol/10^6 cells)	
	After exposure of MOLT-3 cells to 0.3 μg VCR/ml	After exposure of MOLT-3/VCR_{10} cells to 100 μg VCR/ml	After exposure of MOLT-3 cells to 1 μg DXR/ml	After exposure of MOLT-3/$VCR_{1,000}$ cells to 300 μg DXR/ml
10^6	2.6	87.1	45.0	1,930
10^8	0.54	71.7	2.1	1,640

concentrations alone were the determinant of the inoculum effects. The results of similar studies carried out using DXR are shown in Fig. 3. When higher concentrations of drugs were used, the dose-response curves obtained for cell populations of high and low density became close to each other, and the inoculum effects virtually disappeared.

Cellular content of VCR or DXR

Data on the intracellular drug content after 1 h exposure of cells at high and low densities in the pH-controlled medium are shown in Table 1. Exposure of the parent MOLT-3 cells to 0.3 μg VCR/ml (a 1-log cell-kill concentration at the cell density of 10^6/ml) for 1 h resulted in intracellular drug accumulation of 2.6 pmol/10^6 cells when cell density was low, whereas identical experiments conducted at the high cell density resulted in intracellular VCR accumulation amounting to only one-fifth of the low-cell density value. In drug-resistant cell lines, higher concentrations of drugs are needed for the construction of dose-response curves. In MOLT-3/VCR_{10} cells, the ID_{50} value for VCR was approximately 100 μg/ml. When this high drug concentration was used, the cellular VCR contents were virtually identical, irrespective of the cell density.

The cellular contents of DXR were measured in a similar manner. When low concentrations of the drug were used, the cellular DXR content measured at the high cell density was only 5% of that determined at the low cell density. When high concentrations of DXR were used, the cellular DXR contents obtained at high and low cell densities became nearly identical.

The decreased cellular accumulation of both VCR and DXR observed in the sensitive parent cells at the high cell density paralleled the positive inoculum effect. In contrast, when high concentrations of the drugs were used in drug-resistant sublines, no difference in cellular drug accumulation was noted between low and high cell densities and under these conditions the inoculum effects virtually disappeared.

Discussion

In our study of the effect of pH in the medium, we confirmed in two sets of experiments that at acidic pH, the cytotoxic effects of both VCR and DXR were decreased. At the high cell density, the pH of the medium decreased from 7.2 to 6.3, and the dose-response curve moved to the right side of the similar curve constructed at a constant pH

of 7.2. Second, when the pH of the medium was adjusted to 6.3 with acetic acid, the dose-response curve moved to the right, even at the low cell density (1×10^6 cells/ml). However, these adjustments of pH alone did not eliminate the inoculum effects, indicating that pH plays at the most only a minor role. In addition, acidic pH has been reported to increase DDP's activity [7]; however, adjustments of the pH of the medium within the range studied neither influenced the shape of DDP's dose-response curves nor produced positive inoculum effects. Hypoxia might be an issue in the cultures at high cell densities; however, in our experiments, cells were incubated for only 1 h under oxygenated conditions. Acutely hypoxic cells have been reported to be hardly DXR-resistant as compared with chronically hypoxic cells [5]. Furthermore, since the cells we used show population-doubling times of more than 24 h, it is unlikely that a 1-h incubation would produce significantly differential cell-cycle distributions between cells at low versus high densities as the explanation of the inoculum effect.

The present study clearly demonstrates that the relationship between drug concentration and cell density is the major determinant for the inoculum effects seen in VCR- and DXR-induced cell killing. The data obtained in the drug-accumulation studies were consistent with the cell killing. This observation also explains why DDP belongs to a group of drugs that do not produce inoculum effects. On a molar basis, the drug concentrations needed to generate dose-response curves for agents producing positive inoculum effects were generally lower than the required DDP concentrations, e.g., the ID_{90} values for VCR, DXR, and DDP in the parent MOLT-3 cells at a density of 10^6 cells/ml were 0.26, 1 and 27 μM, respectively.

For drugs that produce inoculum effects, there does not appear to be enough drug available to affect all the binding or target sites when cell densities are high, the result being that more cells remain unaffected. Whereas both DDP and DXR are known to intercalate with DNA, DXR also binds to cell-membrane and cytoplasmic components as likely targets for its biological activity [15]. Therefore, each cell appears to contain more binding sites for DXR than for DDP. MOLT-4 cells have been reported to possess vinca-alkaloid receptors at 6×10^6 sites per cell [14], which may be converted to 6×10^{14} sites for 10^8 cells/ml cell suspension. As 1 μg/ml (or 1.0845×10^{-6} M) VCR corresponds to approximately 6.53×10^{14} VCR molecules/ml, if one consideres the cell membrane and other biological barriers the drug must cross to reach tubulin protein, 1 μg VCR molecules appears to be insufficient to saturate all the receptor sites when a high density of cells are used. In this context, the lack of adequate drug molecules to saturate all the tumor cells' drug-binding (or target) sites appears to be the cause of the inoculum effect.

Although we identified the molar drug concentration relative to the cell density as the major determinant of the inoculum effects seen in VCR- and DXR-induced cell killing, we did not conclude that the relative drug concentration is the sole determinant. A more complex process encompassing drug uptake and intracellular drug transport and efflux as variables may contribute to the inoculum effect.

Whereas in vitro data on tumor cells alone are insufficient to enable extrapolation to the biological behavior of a drug in vivo, the inoculum effects appear to be an additional facet of drug resistance for certain chemotherapeutic agents.

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