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Emergence of resistance to VP-16/cisplatin chemotherapy: study in a lymphoblast model system

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Etoposide (VP-16) and cisplatin are widely used in the treatment of malignancy. It is a common clinical observation that patients may initially respond to this two drug combination but later become resistant to it. Data from the CCL-159 lymphoblast cell line suggests that the emergence of resistance may be by means other than multiple drug resistance gene expression. The data suggest that chemotherapeutic failure may be mediated in part by a relatively inefficient method of drug resistance, the unstable expression of low-level drug resistance by a minority of cells. These results may help explain how patients whose malignancies are initially sensitive to VP-16/cisplatin later develop drug resistance.

Key words: Cisplatin, drug resistance, drug therapy, etoposide, leukemia.

Introduction

In recent years the drugs etoposide (VP-16) and cisplatin have both come into wide clinical use in both solid and hematologic malignancies. The two drugs are used in combination in many clinical settings such as the treatment of small cell lung cancer. The small cell lung cancer model demonstrates that patients will often show a dramatic initial response to cisplatin/etoposide combination chemotherapy even though most of these remissions will not be durable. There is relatively little data in the literature exploring the reasons why failure of the etoposide/cisplatinum combination should occur.

Many cases of chemotherapeutic failure have been ascribed in the past to expression of the multiple drug resistance (MDR) phenotype. MDR gene expression has been described in the case of etoposide as well as many other naturally occurring compounds or their derivatives. MDR appears to be only a partial explanation even for etoposide resistance and has generally not been regarded as an important factor in the case of cisplatin resistance.[1,2] In the case of human multiple myeloma, a tumor that is often responsive to chemotherapy but rarely cured by it, mechanisms other than MDR appear to explain at least part of the drug resistance.[3,4] It was of interest for all these reasons to look at how drug resistance to etoposide/cisplatin combination chemotherapy might emerge in a laboratory setting and to explore the nature of multiple drug resistance gene expression in this setting.

The strategy employed in these experiments was to first select for resistance to VP-16. Progeny of survivors of VP-16 exposure were then subjected to another selection process, this time for resistance to cisplatin. The survivors of this two-step selection process (first for VP-16 resistance and then for cisplatin resistance) could then be studied for the stability of their resistance to each of these two chemotherapeutic drugs.

Materials and methods

CCL-159 lymphoblast cells and HL-60 myeloid leukemia cells were obtained from the American Type Culture Collection, and were maintained in a 37°C 5% CO_2 humidified incubator in RPMI 1640 medium with 20% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. Cells were periodically subcultured and cell counts measured by hemocytometer, with percent viability calculated by Trypan blue exclusion, to allow for calculation of the number of viable cells per cubic millimeter.

Cells were exposed to VP-16 and/or to cisplatin either as a continuous drug exposure or as a 4 h pulse exposure. In order to remove drugs, cells would be spun down and resuspended in fresh medium.

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Data on flow cytometric characteristics of the CCL-159 cell line were studied on a FACStar flow cytometer (Becton-Dickinson) equipped with FACStar Plus research software. In some runs, data was gated by use of forward scatter and side scatter criteria. Some runs further used two-color analysis with propidium iodide exposure prior to fixation (to detect dead cells) along with staining (following paraformaldehyde fixation) with an antibody to the MDR protein conjugated to fluorescein isothiocyanate (FITC). (P-glycoCHEK C219; Centocor). Quadrants were set by comparison to samples showing non-specific fluorescence in order to identify the percent of cells falling in the lower right quadrant, showing viability (low prodidium iodide uptake and therefore low fluorescence in channel 2) and high MDR expression (high fluorescence in channel 1). Those cells meeting the forward scatter and side scatter criteria, which had low red fluorescence but high green fluorescence, would be the viable single cells which had overexpression of the product of the MDR gene.

In order to derive a mathematical model for the growth course, the Slide Write Plus (Advanced Graphics Software, Sunnyvale, CA) statistical package was used to compute the least squares regression fit of the growth course for the first week to an exponential formula $y = a_0 e^{a_1 x}$, where y is the viable cell count and x is the number of days. Growth course calculations were based on the initial 7 days of counts to verify that early cell growth of the control cultures followed an exponential model. Exponential growth is shown visually by a linear rise of log viable cell count with time. The effect of chemotherapy exposure on this pattern of exponential growth was evaluated by comparison with the exponentially growing control cells.

Results

Breeding CCL-159 for drug resistance

In initial experiments on wild-type CCL-159 lymphoblast cells, they were able to grow in VP-16 at 0.1 μg/ml continuous exposure but not in 0.5 μg/ml VP-16. In the case of cisplatin, the CCL-159 cells were able to grow in 1 μg/ml but not in 2 μg/ml. By way of comparison, the HL-60 myeloid leukemic cell line (data not shown) had similar VP-16 sensitivity to that of the CCL-159 line, but HL-60 was somewhat more sensitive to cisplatin (unable to grow in 1 μg/ml cisplatin) than was the CCL-159 line.

CCL-159 cells on prolonged exposure eventually yielded some progeny that grew through VP-16 at 2 μg/ml (VP-16 resistant cells), and some of these cells were then used to breed sequentially for resistance to increasing concentrations of cisplatin. Sequential exposures to cisplatin eventually yielded CCL-159 cells that were able to grow slowly despite the presence in the culture medium of cisplatin at 25 μg/ml (double resistance cells).

VP-16 resistance

Several sets of experiments were carried out to characterize the VP-16 effect on CCL-159 cells whose ancestors had undergone selection first for VP-16 resistance followed by a selection for cisplatin resistance. In drug-free conditions, the cells which had undergone this additional selection for cisplatin resistance grew somewhat more slowly than did wild-type cells or than cells which had been selected for resistance to VP-16 alone. Nevertheless, under drug-free conditions the viable cell counts during the first week in culture were well-modeled by exponential growth (R values in excess of 0.9).

Control CCL-159 cells, CCL-159 cells selected only for VP-16 resistance and double resistance cells (Figure 1a, b and c, respectively) all showed some modest decrease in the rate of cell growth with a 4 h exposure to 0.5 μg/ml of VP-16. The cells selected for double resistance, however, showed little more toxicity for 2 μg/ml VP-16 than they did for the 0.5 μg/ml VP-16 dose, while for the wild-type and VP-16 resistance cell lines, there clearly was further toxicity when the pulse VP-16 dose was raised to the 2 μg/ml level. The ability of each of the three cell lines to tolerate pulse VP-16 exposure was incomplete, however, since a 4 h exposure to VP-16 at 10 μg/ml yielded no viable cells for any of the three types of cells even when the cultures were examined 17 days after the drug was removed.

Subsequent experiments were designed to compare the VP-16 effect on the doubly resistant cell line (cells selected sequentially for drug resistance first to VP-16 and then to cisplatin) with the VP-16 effect on wild-type cells. These experiments used VP-16 at a fixed concentration of 2 μg/ml while varying the length of exposure. (None versus 4 h versus 24 h.) Pulse drug exposure was begun at time 0, with cells resuspended in drug-free media after the chosen drug-exposure interval. In these data, as shown (Figure 2a and b), the wild-type cells and the doubly resistant cell line showed similar rates of cell growth in drug free conditions

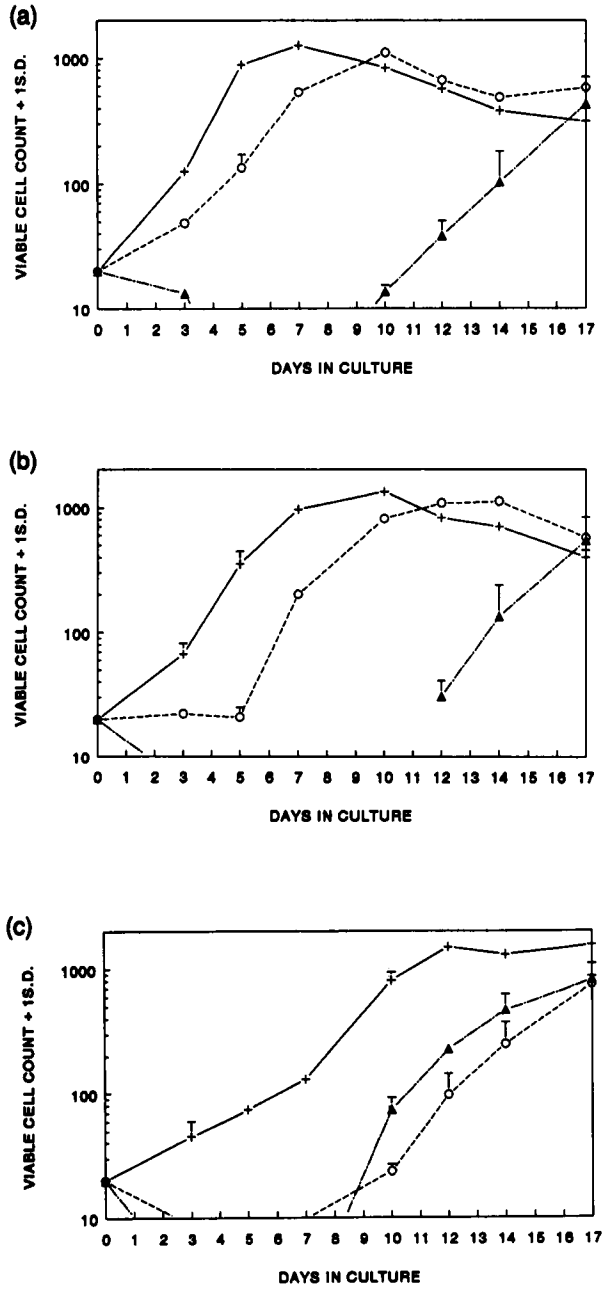


Figure 1. Viable cell counts with 4 h pulse VP-16 (0–10 μg/ml) exposure. (There was no growth at 10 μg/ml.) (a) Wild-type cells, (b) VP-16 resistant cells and (c) double resistance cells. Mean viable cell count for growth of wild-type cells, cells resistant to VP-16 and double resistance cells. The graph error bars show +1 SD on triplicate cultures. +, None; ○, 0.5 μg/ml; ▲, 2 μg/ml.

Exponential modeling:

Growth for the first week of the control cultures (those with no VP-16 added during the 4 h pulse exposure period) was fitted to an exponential model, $y = a_0^{a_1 x}$ and the goodness of the fit indicated by the R value (with 1.0 representing an ideal fit).

Exponential model for initial growth in drug-free media:

$$y = a_0 e^{a_1 x}$$

$$(\ln y = \ln a_0 + a_1 x)$$

	a_0	a_1	R
Wild-type	21.73	0.629	0.979
VP-16 resistant	17.15	0.572	0.990
Double resistance	20.08	0.266	1.000

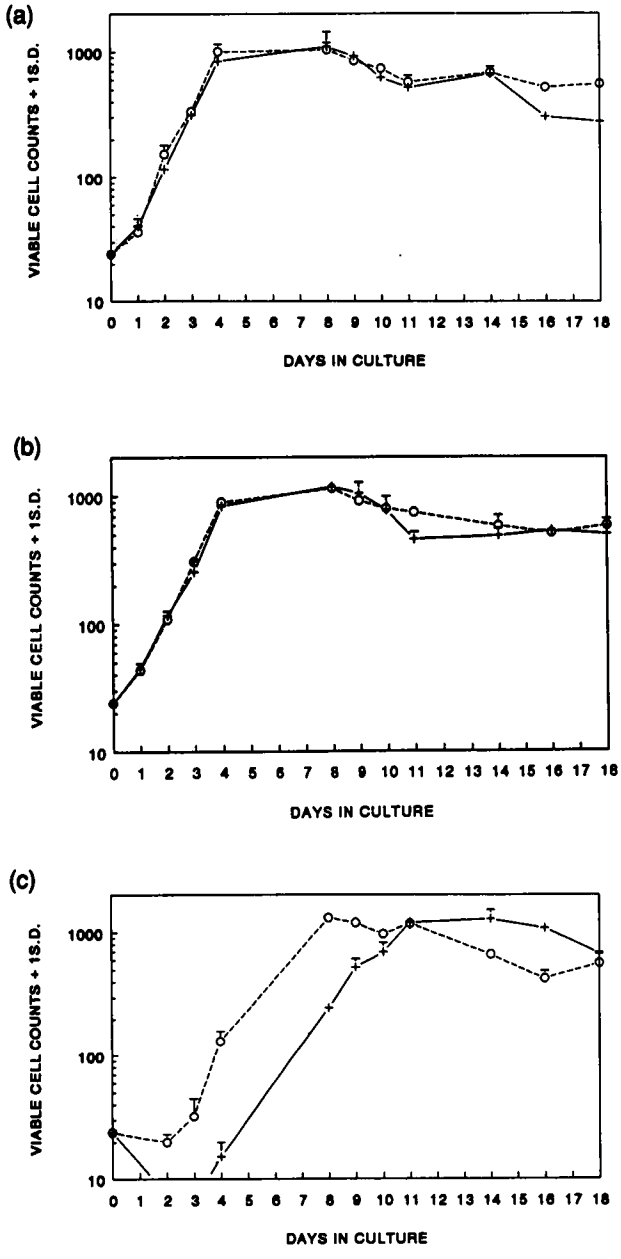
or with a 4 h exposure to VP-16 at 2 μg/ml. (At this point, after some 2 months in culture following the previously described experiment, the progeny of the drug resistance selection now appear to grow about as rapidly under drug-free conditions as did the wild-type cells.) As the period of drug exposure was increased to periods of 24 h (Figure 2c) and 72 h, the VP-16 demonstrated a more pronounced effect, with the rate of recovery of the wild-type cells being much delayed compared with that of the doubly resistant line. In other experiments, 1 week drug exposure to VP-16 at 2 μg/ml sterilized the wild-type cells, while delayed growth was apparent with the doubly resistant line.

Cisplatin resistance

In order to evaluate cisplatin resistance, wild-type and doubly resistant cells were exposed to cisplatin at 2 μg/ml for varying time intervals. The 4 h exposure to cisplatin for either cell line only produced a delay of approximately 2–3 days in the pattern of cell growth (Figure 3). A 24 h exposure in cisplatin was far more toxic, sterilizing all three of the putatively doubly resistant cultures and two of the three wild-type cultures. A single one of the three wild-type cultures showed growth after 2 weeks in drug-free media and this sample (cisplatin resistant cells) was used in further experiments as described below. (Exposures to cisplatin for 48 h or more reliably sterilized both wild-type and doubly resistant cultures.)

Characterization of cisplatin resistance

A final set of cell growth experiments looked at the cisplatin resistant cells (the survivors of the plati-



num exposure from the previous experiment) in comparison to wild-type cells. These survivors of exposure to cisplatin at 2 μg/ml × 24 h had been grown in drug-free media for 1 month, then re-exposed (in triplicate) to either control conditions, cisplatin at 2 μg/ml × 24 h, VP-16 2 μg/ml × 24 h or combination therapy with VP- 16 and cisplatin. Despite the history of resistance to cisplatin the month before, the progeny cells now proved quite sensitive once again to cisplatin (either by itself or combined with VP-16).

MDR expression of these isolated survivors was studied by flow cytometry. The data suggests that, in this previously cisplatin resistant line, MDR expression was more efficiently increased by re-exposure to the two-drug combination than it was by either drug alone. Even with combination therapy, however, only about 3% of the cells detected proved to be viable high-MDR cells.

Discussion

The data reported here suggest that chemotherapy exposure could select for resistance to VP-16 and cisplatin. Resistance was only relative and appeared capable of being overcome by escalations of dose intensity (drug concentration and/or length of exposure). The resistance to cisplatin, in particular, appeared to be a relatively unstable characteristic that could be lost upon growth in the absence of drug. The mechanism(s) of resistance involved are unclear, but it is of interest that the selection for cisplatin resistance appeared to also promote continued VP-16 resistance by the doubly resistant cell line.

Figure 2. Wild-type (+) versus double resistance (○) cells for VP-16 (2 μg/ml) exposure. (a) Control (no VP-16 exposure), (b) 4 h exposure to VP-16 (2 μg/ml) and (c) 24 h exposure to VP-16 (2 μg/ml).

Exponential modeling (details as in legend to Figure 1):

(a) Exponential modeling for initial growth (no VP-16 exposure):

	a_0	a_1	R
Wild-type	19.83	0.917	0.995
Double resistance	19.43	0.969	0.991

(b) Exponential model for initial growth, 4 h exposure to VP-16 at 2 μg/ml:

	a_0	a_1	R
Wild-type	20.74	0.884	0.995
Double resistance	19.93	0.920	0.995

(c) Exponential model for initial growth, 24 h exposure to VP-16 at 2 μg/ml:

	a_0	a_1	R
Wild-type	16.89	−0.167	0.470
Double resistance	16.37	0.371	0.739

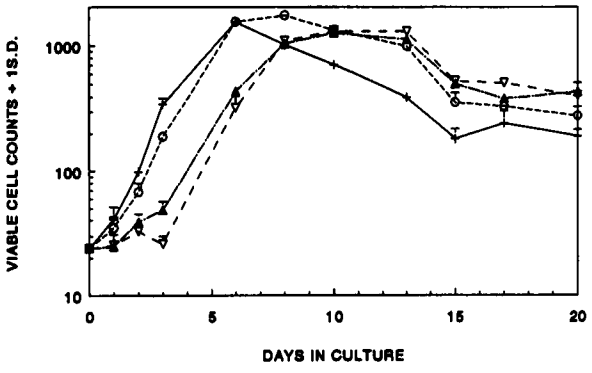


Figure 3. Wild-type and double resistance cells were grown under either control conditions (wild-type (+), double resistance (○)) or with 4 hr exposure to cisplatin 2 μg/ml (wild-type (▲), double resistance (▽)).

Exponential modeling (details as in legend to Figure 1):

Cells	Exposure	a_0	a_1	R
Wild-type	control	24.71	0.719	0.985
Resistant	control	19.68	0.721	0.995
Wild-type	cisplatin	16.40	0.503	0.963
Resistant	cisplatin	15.60	0.435	0.894

Perhaps of most importance is the fact that for either or both the chemotherapeutic drugs in either the wild-type or the cisplatin resistant cell line, only a small minority of cells gated to the lower right (viable cell with high MDR) quadrant. It suggests that this MDR expression is relatively weak and/or unstable, and corresponds to the findings on cell counts in culture suggesting that the resistance characteristic might only manifest its effects after counts are followed for a prolonged time under drug-free conditions. (This clinically would correspond to the possibility of tumor cell regrowth from a resistant subpopulation during the interval between successive cycles of cancer chemotherapy.) It also suggests that mechanisms other than MDR may be of primary importance in the failure of VP-16/cisplatin combination chemotherapy.

Etoposide and cisplatin are two chemotherapeutic drugs that are widely used in clinical oncology. In the experiments reported here the drugs were used sequentially, but in clinical practice they often are given simultaneously.[5,6] It is well known that patients treated with etoposide/cisplatin combination chemotherapy for chemosensitive malignancies such as small cell carcinoma of the lung will often show an initial good response, but then relapse with disease resistant to chemotherapy. Any model for understanding the emergence of clinical drug resistance must consider how a patient can become simultaneously resistant to drugs as dissimilar as etoposide and cisplatin.

The effect of a given drug may be a complicated function of drug concentration and length of drug exposure time. In the case of men with germ cell tumors receiving combination chemotherapy, including 5 days in a row of cisplatin, a doubling of the cisplatin dose increased the toxicity without apparent increase in therapeutic effect.[7] For etoposide, high doses of the drug[8] and prolonged exposures[9] have both been explored.

Etoposide resistance *in vitro* has at times been related to P-glycoprotein overexpression via *mdr-1* gene expression, but this has not been a consistent finding.[10] Many cases of etoposide resistance may reflect instead an atypical form of MDR, acting by means of altered topoisomerase II.[11] Changes in topoisomerase II may also be relevant to cells' ability to tolerate agents as varied as adriamycin[12] and radiation,[13] and suggest the potential relevance of this enzyme to the design of strategies for leukemia therapy.[14]

Conclusion

The data obtained on the CCL-159 lymphoblast cell line suggest that resistance to etoposide and cisplatin in this model may be mediated by mechanisms other than P-glycoprotein, and that the ability to resist chemotherapy is not totally inherited by the progeny of drug-exposed cells. This may be a useful model for better understanding in the typical clinical course of a patient with acute leukemia in relapse, whose tumor may respond transiently to reinduction chemotherapy but who is seldom cured, because of the leukemic cells which survive reinduction chemotherapy. *In vitro*, a variety of drug combinations, drug sequence, and duration and intensity of drug exposure could be explored in a model of this type to try to improve therapeutic efficacy. This may also suggest strategies for use of platinum based compounds (such as perhaps carboplatin[15]) either alone or combined with drugs such as etoposide in leukemia patients.

References

1. Gately DP, Howell SB. Cellular accumulation of the anticancer agent cisplatin: a review. *Br J Cancer* 1993; **67**: 1171–6.
2. Scanlon KJ, Kashani-Sabet M, Tone T, *et al.* Cisplatin resistance in human cancers. *Pharmacol Ther* 1991; **52**: 385–406.

3. Sonneveld P, Durie BGM, Lokhorst HM, *et al.* Analysis of multidrug-resistance (MDR-1) glycoprotein and CD56 expression to separate monoclonal gammopathy from multiple myeloma. *Br J Haematol* 1993; **83**: 63–7.
4. Ishikawa H, Kawano MM, Okada K, *et al.* Expressions of DNA topoisomerase I and II gene and the genes possibly related to drug resistance in human myeloma cells. *Br J Haematol* 1993; **83**: 68–74.
5. Goodman GE, Crowley J, Livingston RB, *et al.* Treatment of limited small-cell lung cancer with concurrent etoposide/cisplatin and radiotherapy followed by intensification with high-dose cyclophosphamide. *J Clin Oncol* 1991; **9**: 453–7.
6. Kirmani S, Lucas WE, Kim S, *et al.* A phase II trial of intraperitoneal cisplatin and etoposide as salvage treatment for minimal residual ovarian carcinoma. *J Clin Oncol* 1991; **9**: 649–57.
7. Nichols CR, Williams SD, Loehrer PJ, *et al.* Randomized study of cisplatin dose intensity in poor-risk germ cell tumors. *J Clin Oncol* 1991; **9**: 1163–72.
8. Reece DE, Barnett MJ, Connors JM, *et al.* Intensive chemotherapy with cyclophosphamide, carmustine and etoposide followed by autologous bone marrow transplantation for relapsed Hodgkin's disease. *J Clin Oncol* 1991; **9**: 1871–9.
9. Greco FA, Johnson DH, Hainsworth JD. Chronic oral etoposide. *Cancer* 1991; **67**: 303–9.
10. Long BH, Wang L, Lorico A, *et al.* Mechanism of resistance to etoposide and teniposide in acquired resistant human colon and lung carcinoma cell lines. *Cancer Res* 1991; **51**: 5275–84.
11. Beck WT. Strategies to circumvent multidrug resistance due to P-glycoprotein or to altered DNA topoisomerase II. *Bull Cancer* 1990; **77**: 1131–41.
12. Deffie AM, Bosman DJ, Goldenberg GJ. Evidence for a mutant allele of the gene for DNA topoisomerase II in adriamycin-resistant P388 murine leukemia cells. *Cancer Res* 1989; **49**: 6879–82.
13. Cunningham JM, Francis GE, Holland MJ, *et al.* Aberrant DNA topoisomerase II activity, radioresistance and inherited susceptibility to cancer. *Br J Cancer* 1991; **63**: 29–36.
14. Zwelling LA. Topoisomerase II as a target of antileukemia drugs: a review of controversial areas. *Hematologic Pathol* 1989; **3**: 101–12.
15. Lee E, Van Echo DA. The potential of carboplatin in human acute leukemia. In: Bunn PA, Canetta R, Ozols RF, *et al.*, eds. *Carboplatin (JM-8)*. Philadelphia: WB Saunders 1990: 361–5.

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